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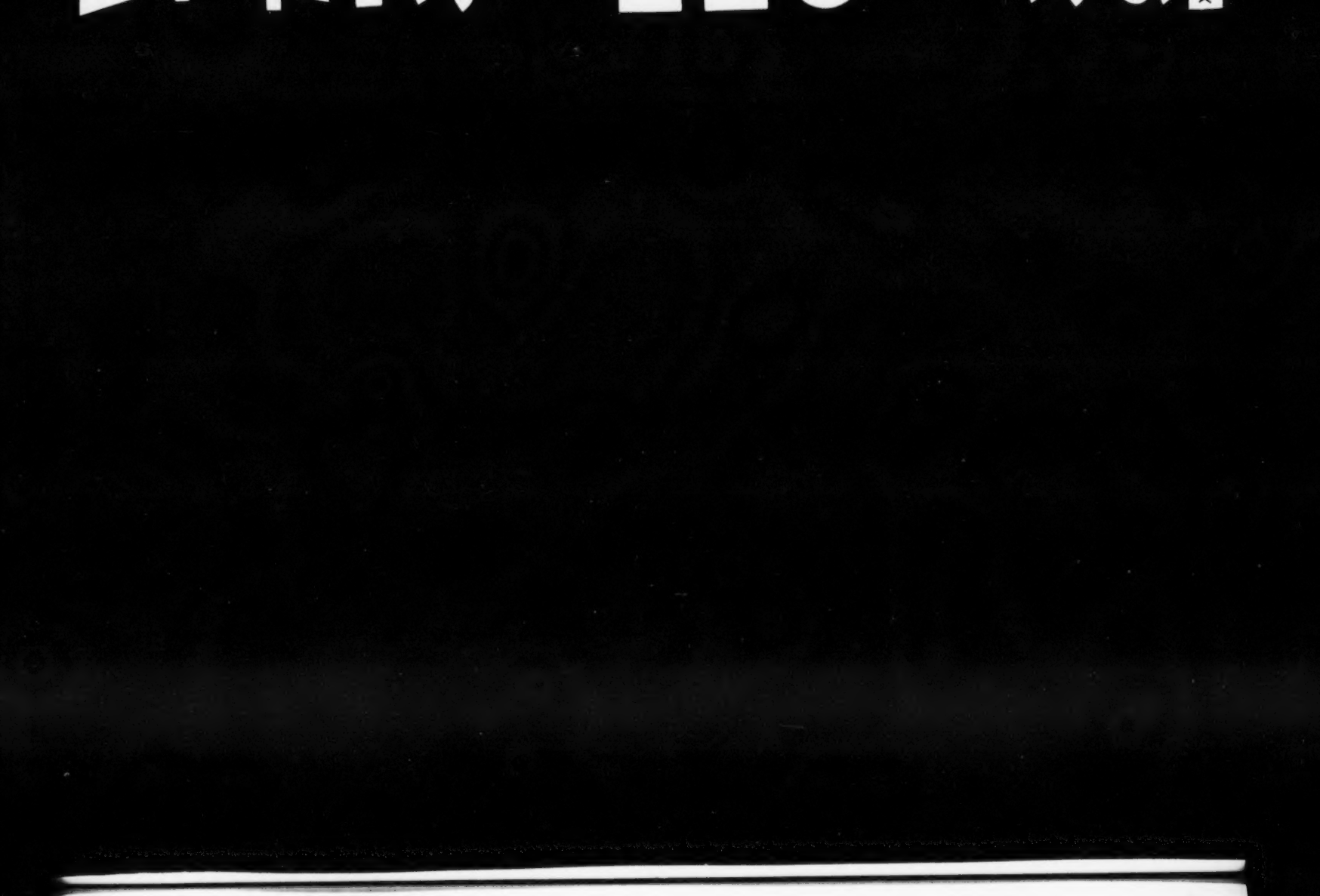

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THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE EFFECTS OF A LACK OF OXYGEN AND OF LOW OXYGEN TENSIONS ON PARAMECIUM

J. A. KITCHING[1]

(From the Department of Physiology, Princeton University, Princeton, N. J., and the Department of Zoölogy, University of Bristol, England)

Introduction

The ability of *Paramecium* to survive without oxygen has been the subject of many investigations, and the results previously published are at variance with one another. Loeb and Hardesty (1895) confined *Paramecium* in a special gas chamber which they freed of oxygen either with a stream of hydrogen or by means of alkaline pyrogallol; the organisms died after twenty-four hours. Budgett (1898), using an Engelmann gas chamber flushed with hydrogen, obtained a survival time of several hours, after which the *Paramecium* blistered. Pütter (1905) found *Paramecium caudatum* to survive for five or six days in a specially designed bottle which had been flushed thoroughly with nitrogen. Fauré-Fremiet et al. (1929) got a survival time of twenty-four hours at 25° C. in sealed tubes containing leuco-methylene blue (reduced by sodium hydrosulphite). Juday (1909) discovered *Paramecium* sp. in the deeper waters of Lake Mendota (Wisconsin) at a time when he believed these waters to be devoid of free oxygen; according to him *Paramecium* is therefore able to live anaerobically for several months. Fortner (1924) enclosed *P. caudatum* in an atmosphere of hydrogen together with aerobic bacteria to remove any remaining oxygen; they survived for several hours, and their contractile vacuoles continued to function, although at a reduced frequency. Gersch (1937) found that *Paramecium* died within 10 seconds in gas purified of its oxygen by means of alkaline pyrogallol. In the work which is described in this paper I have attempted to harmonize these discrepancies.

In addition it has been claimed by several investigators that the respiration of *Paramecium* is insensitive to cyanide (Lund, 1918; Gerard and Hyman, 1931; Shoup and Boykin, 1931). I have therefore

[1] Fellow of the Rockefeller Foundation.

made some observations to determine the capacity of *Paramecium* to carry on its normal activities, such as secretion by the contractile vacuoles and locomotion, in the presence of cyanide.

Apparatus and Methods

Anaerobiosis in a Hanging Drop

In subjecting Protozoa to a lack of oxygen, it is necessary to ensure the following conditions:

(1) A genuine lack of oxygen. It is not possible to assure complete absence of oxygen molecules, but the oxygen content of the medium surrounding the organisms must be so small that the organisms could not possibly make any significant use of it for oxidative metabolism. Oxygen must not be generated by accompanying plant cells or bacteria.

(2) No extraneous contamination. For instance, there must be no harmful impurities in any gas used. For this reason the absorption of oxygen by alkaline pyrogallol is perhaps to be avoided.

(3) No harmful secondary effects. For instance, the flushing of the medium with an oxygen-free gas mixture must not result in a harmful shift of the hydrogen ion concentration.

The general method used was to pass oxygen-free gas past a hanging drop containing the organisms. For this purpose cylinder hydrogen, or cylinder nitrogen (water-pumped), was first bubbled through concentrated sulphuric acid, dilute sulphuric acid, dilute potassium hydroxide and distilled water. Next it was purified of its oxygen. In most experiments hydrogen was used, and was passed through an electrically heated quartz tube containing platinized asbestos. The quartz tube extended sufficiently far at each end beyond the heating coil to avoid any significant warming of the deKhotinsky cement seals. In some experiments, however, nitrogen was passed over hot copper in a special internally heated furnace described by Kendall (1931). This furnace consists of a Pyrex glass tube containing a cylinder of copper gauze of large surface area, which is heated from within by means of a coil of nichrome wire. In this furnace it is always possible to see the condition of the copper, and in the time required for one experiment only a small section of the copper, at the inflow end, became tarnished. Finally the gas was carried by pure lead tubing with seals of deKhotinsky cement through a closed glass wash tube with distilled water in it to the chamber containing the organisms.

The organisms were mounted in a hanging drop on a coverglass which was sealed with vaseline or a mixture of vaseline and paraffin

wax to the chamber. The hanging drop was not allowed to touch the vaseline. The chamber itself consisted of a glass ring about 2 cm. in diameter and 1 cm. deep, closed underneath by a microscope slide to which it was sealed with deKhotinsky cement, and with glass inlet and outlet tubes. The upper edge of the ring was ground to support a coverglass, and the microscope slide which formed the base of the chamber fitted onto the mechanical stage of the microscope.

From the chamber the gas was carried by lead tubing to a light-proof box, where it was bubbled either through a simple water trap or through a suspension of marine luminous bacteria. These bacteria (*Achromobacter fischeri*) luminesce in the presence of minute traces of oxygen, and according to Harvey and Morrison (1923) about 0.005 mm. of oxygen can be detected in this way. After the purified hydrogen or nitrogen had bubbled for five or ten minutes through the bacterial suspension no luminescence could be detected with the dark-adapted eye, but admission of air gave immediate recovery of luminescence. An analysis of the purified gas made by mass spectrograph by Dr. W. R. Guyer showed no trace of oxygen, although one part in 10^5 could have been detected.

For control experiments another glass chamber, similar to the first, was mounted on the stage of a second microscope, and CO_2-free air was drawn through gently with a suction pump.

Anaerobiosis in a Tube with Reduced Redox Indicator

The survival of organisms in the presence of a reduced redox indicator low in the redox scale has been used as a demonstration of anaerobic life. For instance, Clark (1924) found that certain bacteria reduced indigo carmine until a high ratio of the reduced to the oxidized substance was attained. He calculated that the oxygen tension in equilibrium with this mixture was so low as to be physically meaningless. For the present purpose, however, such a condition does not establish anaerobiosis, since the bacteria were obtaining oxidative energy by reducing the dye. The dye should therefore be reduced as completely as possible to avoid this possibility. Furthermore, the use of methylene blue, indophenols, or other indicators high in the series is to be avoided. The reoxidation of indicators high in the series is relatively slow at atmospheric oxygen tension (Barron, 1932), and might be very slow at low oxygen tensions even at the experimental hydrogen ion concentration. Harvey (1929) has shown that indophenols in the presence of luminous bacteria may remain reduced in the presence of a concentration of free oxygen sufficient to allow the bacteria to luminesce. Under certain conditions equilibrium may never be attained, and the state of

the dye is then no indication of the oxygen tension in the solution. The method described below to the best of my knowledge avoids these errors.

Paramecium in the requisite medium was placed in an internally sealed glass wash tube with some platinized pumice and sufficient indigotrisulphonate to color the solution a clear blue. (Platinized pumice was found more satisfactory than platinized asbestos, as the latter is easily stirred up, and splits into fine sharp spikes which may damage the organisms. Platinized pumice was prepared by boiling pumice chips in chloroplatinic acid until most of the air had been driven out of the pores. Then sodium formate was added until platinum was precipitated in the pores and all over the surface of the pumice. The chips were then washed very thoroughly in running water.) The wash tube was sealed with deKhotinsky cement to the source of purified hydrogen, and the gas escaping from it was carried by lead tubing to the anaerobic chamber for a parallel experiment. The indicator dye bleached five or ten minutes after the hydrogen was turned on. The wash tube was then wrapped completely and thoroughly in black cloth so as to exclude all light, and slow bubbling of the hydrogen was continued. This precaution was taken although no photosynthetic organisms were ever detected in the cultures.

Controlled Oxygen Tensions

In order to subject *Paramecium* to controlled and known oxygen tensions, the apparatus used for anaerobiosis in a hanging drop was modified. Hydrogen, purified of its oxygen as already described, and oxygen were led through flow meters (see Harvey and Morrison, 1923) to a T tap in which they were mixed. The resulting known gas mixture was then conveyed through the wash tube with distilled water to the observation chamber. Lead tubing and seals of deKhotinsky cement were used throughout. According to a later refinement, condensation of water in the capillaries of the flow meters was prevented by a tubular show-case lamp placed alongside them. By manipulation of the T tap the organism could be subjected rapidly either to pure hydrogen or to any desired ratio of hydrogen and oxygen.

In a few experiments at very low oxygen tensions, oxygen was mixed with hydrogen in proportions measured by flow meters, and a small part of the resulting mixture was then mixed in the same way with additional pure hydrogen. The unwanted part of the first mixture was rejected through a mercury trap. In this way extremely low tensions of oxygen could be provided with reasonable accuracy.

The tension of oxygen in the observation chamber was calculated

with due regard for the barometric and water vapor pressures. The total gas pressure in the observation chamber exceeded that of the air by an insignificant amount.

The Diffusion of Oxygen in a Hanging Drop

I am indebted to Dr. H. P. Robertson of the Physics Department, Princeton University, for a mathematical expression relating the thickness of the hanging drop, the original concentration of oxygen in it, the diffusion coefficient of oxygen in water, and the time required to bring that drop to any given low oxygen tension after the drop has been placed over an atmosphere devoid of oxygen. The drop has been regarded as a film parallel to the coverglass, which for my experiments is reasonably true. Of the infinite series which was derived only the first term is significant, viz.

$$t \sim -\frac{4b^2}{\Delta\pi^2} \cdot \log_e \frac{\theta\pi^2}{8},$$

where t = the time required for the equilibration,
b = the thickness of the drop,
θ = the ratio of concentration of oxygen attained at time t to initial concentration,
Δ = the diffusion coefficient of oxygen in water.

For conditions approaching those of the experiments Δ is given by Bruins (1929) as very nearly 2.0×10^{-5}.

It will be seen that the time required for the drop to reach any given oxygen tension varies as the square of its thickness. Let us choose arbitrarily an oxygen tension of $10^{-4} \times$ the oxygen tension of the atmosphere—less than one-tenth the minimal value necessary (as will be shown later) to allow activity in *Paramecium*. For $\theta = 10^{-4}$, when $b = \frac{1}{4}$ mm., $t = 2$ minutes; and when $b = \frac{1}{2}$ mm., $t = 8$ minutes. The films used were of this order of thickness, and in practice it is probable that equilibration is accelerated by the water currents set up by the swimming of the *Paramecium*, as well as by convection currents.

These theoretical results agree well with experiments on certain marine amoebae, to be published later, in which oxygen tensions below $\frac{1}{2}$ mm. are required for stoppage of movement, and which in a hanging drop come to a standstill within five minutes of the time when pure hydrogen is turned on. It may be concluded that in my experiments adequately anaerobic conditions were attained within ten minutes. Similarly the equilibration of a hanging drop with gas mixtures of known low oxygen tension must be rapid, provided always that the drop is a thin one.

Material

Paramecium was cultured in timothy hay infusion, and was obtained from the following sources:

(1) *P. multimicronucleatum* collected from a backwater of the canal near Princeton, N. J. Individuals of this race had three contractile vacuoles.
(2) *P. multimicronucleatum* of a pure line (Clone I), with two contractile vacuoles.
(3) *P. caudatum* of a pure line (Clone D).

Table I

Survival of *Paramecium multimicronucleatum* clone I under anoxic conditions in a hanging drop of its own culture fluid. CO_2, when used, was at a partial pressure of 12 mm., which was sufficient to maintain the culture fluid at pH 7.1 approximately. Room temperature was 24–28°C.

Gas used	Time for first *Paramecium* to blister	Time for fifty per cent to blister	Time for last to blister	Number of animals used	Date (1939)
	minutes	*minutes*	*minutes*		
Pure H_2	160	370	440	14	August 6
Pure N_2	210	465	585	43	August 3
$H_2 + CO_2$	360	650	690	19	July 29
$H_2 + CO_2$	595	665	710	28	August 1
$N_2 + CO_2$	500	660	720	12	August 5
$N_2 + CO_2$*	500	660	725	9	August 7
CO_2-free air (control experiment)	—†	—	—	21	July 30–31

* Extra pure CO_2: see text.

† All survived throughout experiment (31 hours) and remained normal in appearance and activity.

The last two were both kindly supplied to me through the courtesy of Professor S. O. Mast of the Johns Hopkins University.

Results

In all cases and in all media *Paramecium multimicronucleatum* and *P. caudatum*, when mounted in a hanging drop in absence of oxygen, continued to swim and to evacuate fluid by the contractile vacuoles for a limited time. However, the speed of swimming and the rate of output of the contractile vacuoles decreased, and eventually the organisms stopped. Then the trichocysts were discharged; the anterior end of the body usually, but not always, became constricted; blisters formed at the surface of the body; and cytolysis ensued. *Paramecium* which had

ceased all activity for lack of oxygen, and which had even begun to blister, recovered rapidly on admission of sufficient oxygen (see Tables III and IV).

The duration of anaerobic survival was very variable, and depended partly on the media used. The longest survival times were got with culture fluid. In hanging drops of culture fluid survival times in absence of oxygen ranged from one to twelve hours. It appeared (as already shown by Pütter (1905)) that *Paramecium* from well-fed, flourishing cultures survived the longest. There were considerable differences between cultures of the same clone. However, exposure of a hanging drop of culture medium to pure hydrogen or nitrogen leads to

TABLE II

The effect of a lack of oxygen on *Paramecium caudatum* clone D, after segregation in test tube in culture fluid without hay.

Medium	Culture fluid; pure hydrogen	M/300 phosphate buffer; pure H_2	M/300 phosphate buffer; CO_2-free air as control
Experiments on same day:			
Time required for first one to stop or blister, in minutes	88	23	no visible adverse effects
Same for 50 per cent	154	56	—
Same for last one	235	76	—
Number of individuals used	28	8	12
Experiments five days later:			
Time required for first one to stop or blister	79	7	27
Same for 50 per cent	79	7	29
Same for last one	109	9	68
Number of individuals used	8	4	7

a loss of carbon dioxide, with a resulting shift of the hydrogen ion concentration far into the alkaline range. Accordingly, pure hydrogen was bubbled through some culture fluid and the resulting shift of pH, according to measurements kindly made for me with the glass electrode by Dr. Marshall E. Smith, was from about 6.2–6.4 to about 8.9–9.0. Addition of about 12 mm. partial pressure of carbon dioxide to the hydrogen was sufficient to maintain the culture fluid at about pH 7.1. When hydrogen or nitrogen together with this amount of carbon dioxide were passed through the observation chamber, the *Paramecium* survived slightly but significantly longer than without the carbon dioxide (Table I); the series of changes leading to death was, however,

the same. In these experiments no purification of the carbon dioxide was undertaken, but the connection bewteen the carbon dioxide cylinder and the apparatus was entirely of glass, lead tubing, and deKhotinsky cement, and the pressure was regulated by a double water trap of such a nature as to prevent backward diffusion of oxygen. The manufacturers of the carbon dioxide stated that the oxygen content of their cylinders varied between two and sixteen parts in ten thousand. The oxygen content of the mixed carbon dioxide and nitrogen or hydrogen must have been insignificantly low, and experiments with a cylinder

TABLE III

The effect of distilled water, as compared with culture fluid, on the ability of *Paramecium multimicronucleatum* to withstand a lack of oxygen. For each gas mixture two separate hanging drops, with the two media, were suspended from the same coverglass.

Time	Pure Hydrogen		CO_2-free Air (Control)	
	Culture fluid	Distilled water	Culture fluid	Distilled water
minutes				
0	started	started	started	started
57	all very slow	1 blistered, rest very slow	normal movement	normal movement
64	1 blistered, rest very slow	"	"	"
73	all stopped	all stopped	"	"
74	3.5 mm. oxygen admitted			
80	3 swimming, rest appear dead	3 swimming. rest appear dead	"	"
395	2 swimming normally, rest dead	2 swimming normally, rest dead	"	"
Number of organisms used	15	12	12	8

which had been cleaned out and filled with special care by the manufacturers gave entirely similar results.

The results obtained in the presence of a reduced redox indicator (indigo trisulphonate) in a wash tube in the dark agreed well with those obtained by the hanging drop method. In several experiments the purified hydrogen was bubbled first through the wash tube with the platinized pumice, and then through the anaerobic chamber. In all such experiments a few individuals survived in the wash tube after all had cytolysed in the anaerobic chamber. However, the wash tube contained many thousands of individuals, and it is to be expected that out of so many

a few would be more hardy. At the time when the majority in the anaerobic chamber cytolysed there was a marked decrease in the number visible in the wash tube. The longest survival in culture fluid was about twelve hours.

In other experiments the organisms were washed four times with M/300 phosphate buffer ($Na_2HPO_4 + KH_2PO_4$) at pH 7.0–7.1, and mounted over the anaerobic chamber in this medium. Under anoxic conditions *Paramecium* underwent the same series of changes as in culture medium, although the constriction of the anterior end and the blistering seemed more sudden and violent. Also it survived for much less time in phosphate buffer than in culture fluid; even though in air

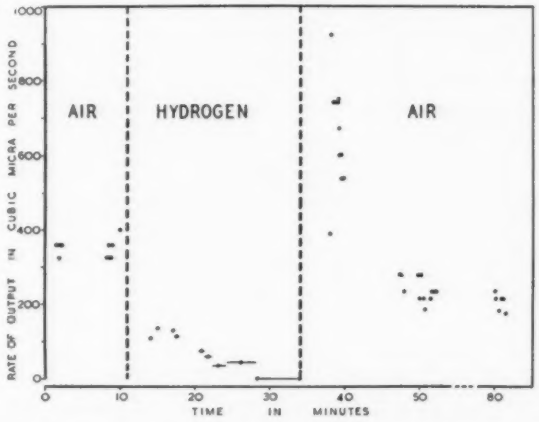

FIG. 1. The effect of pure hydrogen on the rate of output of the anterior contractile vacuole of *Paramecium multimicronucleatum* in a hanging drop of dilute phosphate buffer.

it lived without apparent damage for many hours (in some cases observations were extended over two days) in the buffer solution. However, if some *Paramecium* were removed from the culture and placed in a test tube with some of the culture fluid but without hay, their ability to survive anaerobically in either medium (culture or buffer solution) decreased progressively, and after a few days they became fatally susceptible to phosphate buffer even when in air. These results are illustrated in Table II.

In the absence of oxygen *Paramecium* was found (in a few experiments) to survive equally well either in culture fluid or in distilled water (Table III). However, in M/300 KCl some individuals cytolysed

almost instantly in air, and in absence of oxygen the remainder survived only a few minutes.

The contractile vacuoles, both in culture medium and in phosphate buffer, continued to function under anaerobic conditions, but finally be-

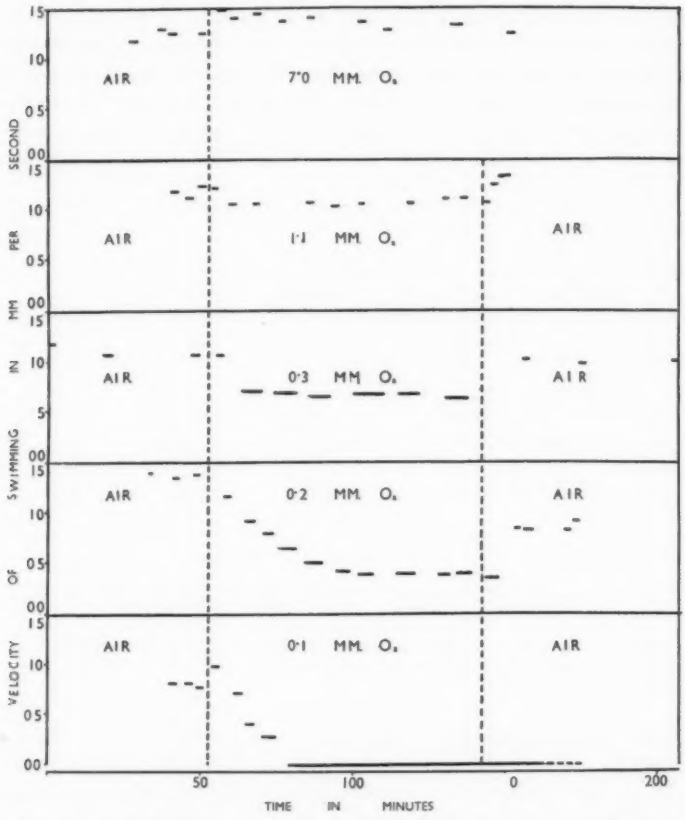

FIG. 2. The influence of low oxygen tensions on the rate of swimming of *Paramecium multimicronucleatum* in dilute phosphate buffer.

came very slow. In two experiments *Paramecium* was slowed down by means of an agar gel just sufficiently viscous to make observation possible. The result of one of these experiments is plotted in Fig. 1. The stoppage of the contractile vacuoles after a period of lack of oxygen was found to be reversible. Contrary to the findings of Frisch (1937),

the *Paramecium* which I used showed normal activity of the contractile vacuoles whether they were swimming or stationary. This was true of *Paramecium* in hanging drops of culture medium or dilute buffer in contact with air, with or (usually) without agar. I attribute the discrepancy to the fact that his organisms had been sealed in a vaseline ring for many days and were not fully active.

A series of experiments was made to determine the survival and activity of *Paramecium multimicronucleatum* at low oxygen tensions. The rate of swimming of healthy *Paramecium* in phosphate buffer was found, after an initial burst of high activity, to remain reasonably constant for as long as observations were continued (up to 22 hours).

TABLE IV

Recovery of *Paramecium caudatum* at known oxygen tensions from lack of oxygen, in dilute phosphate buffer solution.

Duration of lack of oxygen in minutes	Condition at end of anaerobic period: number of animals		Oxygen admitted in mm.	Duration of observations at this tension in minutes	Condition after admission of oxygen at this tension: number of animals		
	Stopped but normal in shape	Stopped but pointed and blistered			Recovered after stoppage	Recovered after stoppage and blisters	Cytolysed
44	1	2	27	36	1	1	1
46	6	2	7.0	38	6	1	1
31	5	2	0.28	21	3	1	3
36	1	7	1.4	38	1	6	1
31	1	5	0.85	41	1	1	4
57	3	2	0.57	22	3	0	2
53	11	4	0.23	24	1*	0	14
36	3	1	0.17	18	0	0	4

* Movement very slow.

Accordingly single organisms were acclimatized for 90 minutes in a hanging drop of the buffer solution in a stream of moist carbon dioxide-free air. After this observations were made of the rate of swimming (*a*) in carbon dioxide-free air, (*b*) in a known mixture of oxygen and hydrogen, and (*c*) in carbon dioxide-free air. The second period (*b*) lasted usually about two hours. Readings of the time required for the organisms to traverse the distance indicated by the ocular scale (2.06 mm.) were made in groups of twelve, and the mean for each group determined. The standard error of the mean time usually lay between 3 and 5 per cent of the mean value. Results are shown in Fig. 2. There was a slowing down of swimming in oxygen tensions below 1 mm. Below 0.2 mm. approximately the organisms died, but above this value

they slowed down to a speed approximately constant within the duration of the treatment; and this effect was reversible. Individual variation was such that it did not seem worthwhile to try to determine a detailed relation between oxygen tension and rate of swimming.

In a further series of experiments a group of *Paramecium* was mounted in a hanging drop of the dilute phosphate buffer in the anaerobic chamber, and subjected to anoxic conditions until all had stopped and some of them had blistered. Then oxygen was admitted at a known partial pressure, and the extent of recovery recorded. Results are shown briefly in Table IV. Recovery could be obtained, on admission of sufficient oxygen, even after the organisms had begun to blister, and in such cases the blisters were gradually resorbed. In many of these cases it is certain that if the organisms had been left for another one or two minutes without oxygen irreversible cytolysis would have ensued. The minimal oxygen tension needed for recovery was of the same order as that which was found just to allow swimming in the previous series of experiments.

The effect of cyanide on the secretory activity of the contractile vacuoles and on the general activity of the animals was examined briefly in three experiments. Single individuals of *Paramecium multimicronucleatum* were mounted in a hanging film of river water just sufficiently thin to prevent too rapid swimming. The cover glass was sealed with vaseline over a small glass cell half filled with river water, so that evaporation from the film was prevented. After a period of examination in river water the *Paramecium* was transferred with the usual four washes to a dilute solution of sodium cyanide (M/200, M/1,000, M/2,000, pH corrected to 7.1) in river water, and the fluid in the cell was also replaced by the cyanide solution. This procedure was carried out as quickly as possible in order to avoid loss of cyanide, and the examination of the organism was continued. After an initial depression of activity the vacuoles continued to function regularly although slightly less vigorously than in plain river water. The cilia also continued to beat actively. Observations were continued in one case for over ten hours after the cyanide treatment was begun.

Discussion

The majority of workers are agreed that *Paramecium* at room temperatures (20–25° C.) can survive for some hours, though not days, without oxygen. In view of the indubitable demonstrations of this fact, the results of Gersch (1937), who observed almost instantaneous death, must be discounted. It seems somewhat questionable whether Pütter, who (1905) obtained a much longer supposedly anaerobic sur-

vival, really achieved strictly anoxic conditions; and it is clear that *Paramecium* can make some use of oxygen at partial pressures below 1 mm. It also seems doubtful whether Juday (1909) would have detected oxygen in such low concentrations in the bottom waters of Lake Mendota (Wisconsin), where he claimed that *Paramecium* lived anaerobically. However, it remains possible that *Paramecium* might be found to survive without oxygen for longer periods under experimental conditions if it were supplied with suitable food. Slight discrepancies between the results of other workers may be ascribed to variations in the excellence of the oxygen "lack," possibly to the use of several different species, and particularly to variations in the state of nutrition of the organisms. This latter condition was stressed by Pütter (1905), and probably accounts for the beneficial effect of stirring the culture.

There is clearly some adverse influence in phosphate buffer which a normal healthy *Paramecium* can withstand in air, but which successfully operates against a starved *Paramecium* in air or against a well-fed one in absence of oxygen. The constriction of the anterior end of the organism and the blistering just before death are probably the result of a violent contraction of the myonemes. They were found to occur even when the organism was in M/20 lactose solution, and it is therefore unlikely that they can be attributed to osmotic uptake of water by the organism. A somewhat similar effect has been seen in *Paramecium* subjected to an electric current (see Kalmus, 1931). The phosphate buffer, either directly or indirectly, hastens the time for the myoneme contraction. It seems probable that the harmful effect of this buffer is due to a lack of balance of ionic concentrations, although this matter has not yet been investigated in detail.

Whereas cyanide in very low concentration inhibits the action of the contractile vacuoles of peritrich ciliates (Kitching, 1936), it has no very marked effect on those of *Paramecium*. The prolonged anaerobic activity of *Paramecium* might account for this. However, according to the results of various workers, at least a considerable part of the respiration of *Paramecium* must be insensitive to cyanide. Gerard and Hyman (1931) found that substitution of phosphate buffer for a calcium-containing water approximately halved the rate of oxygen consumption, but addition of cyanide made no further difference. The persistence of vacuolar activity in the presence of cyanide harmonises with the view that the respiration of *Paramecium* is relatively insensitive to this substance.

Paramecium is able to continue swimming, or to recover from a lack of oxygen, at oxygen tensions down to 0.3 mm. However, it is possible that higher tensions may be needed for growth and prolonged survival.

Amberson (1928), by a not very delicate method, obtained results which suggest that the respiration of *P. caudatum* is depressed slightly at tensions of oxygen below 50 mm.

A comparison of the results obtained with *Paramecium* and with other Protozoa will be made in another paper.

ACKNOWLEDGMENTS

I wish to thank Dr. E. N. Harvey for his guidance and criticism, as well as for the facilities of the physiological laboratory, Princeton University. The work was completed at the Marine Biological Laboratory, Woods Hole.

SUMMARY

1. *Paramecium multimicronucleatum* and *P. caudatum* were subjected to pure hydrogen, pure nitrogen, and known mixtures of hydrogen and oxygen, while in a thin hanging drop under microscopical observation.

2. In all cases there was a limited period of anaerobic survival, during which activity of swimming and of contractile vacuoles was gradually diminished. Finally the organisms stopped, blistered, and cytolysed.

3. Admission of sufficient oxygen, even after blistering had begun, gave recovery.

4. Survival under anoxic conditions was best in culture fluid maintained at a reasonable hydrogen ion concentration by the addition of small quantities of carbon dioxide to the hydrogen or nitrogen. Under these conditions some organisms survived as long as twelve hours.

5. *Paramecium* was extremely variable as regards the length of anaerobic survival in any one medium. This variability is ascribed to physiological condition and not to genetic factors.

6. Survival under anoxic conditions was much shorter in dilute phosphate buffer than in culture medium. This is tentatively ascribed to a lack of balance of ionic concentrations.

7. In phosphate buffer the rate of swimming was reduced at tensions of oxygen below 1 mm., and the organisms died within a short time at tensions below 0.2 mm. At tensions above 0.3 mm. some measure of recovery from lack of oxygen could be obtained.

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DARK ADAPTATION AND REVERSAL OF PHOTOTROPIC SIGN IN DINEUTES

J. E. G. RAYMONT

(From the Biological Laboratories, Harvard University)

Introduction

Animals which exhibit orientation and directed movement with respect to a light source are said to be positively or negatively phototropic, according as they move towards or away from the source. Under certain conditions, a change of phototropic sign may occur, e.g. an animal which normally moves towards the source will move from it. Among the more important factors which have been shown to produce such a reversal of phototropic sign are: difference in absolute light intensity, change of light intensity, temperature, pH, and some chemical substances. The possible effect on phototropic sign of the degree of dark adaptation, however, has been largely neglected. Crozier and Wolf (1928) demonstrated the effect of dark adaptation on the strength of phototropism in *Agriolimax* and more recently Wolter (1936) has reported that some specimens of *Carcinus maenas* show a change of phototropic sign with dark adaptation. Since Clark (1931, 1933) demonstrated the process of dark adaptation by means of phototropic reactions for the "whirligig beetle" (*Dineutes assimilis*), it was decided to use this beetle in testing for the possible effect of dark adaptation on the sign of phototropism.

Material and Methods

The animals used in these experiments were of the species *Dineutes horni*,[1] and were collected from Fresh Pond, Cambridge, Massachusetts. They were kept in an aquarium in a lighted room, and were fed on pieces of fresh meat and fish, floated on wood.

The eye in *Dineutes* is divided into dorsal and ventral parts on each side, and the phototropic responses were compared when the whole, and when only part of each eye was functional. Blinding was effected by blackening the surface of part of the eye with asphaltum varnish.

In each experiment the beetle was first light-adapted and was then left for the desired period in a covered vessel of water. Following this

[1] I am indebted to Mr. C. Parsons of Harvard University for the identification of this species.

period in darkness the phototropic reaction was observed by allowing the animal to crawl on a dry, horizontal test-plate of ground glass. This was illuminated from one side by a diverging beam of light emanating from a slit ($1\frac{1}{2}'' \times \frac{3}{4}''$) covered by a ground glass plate, behind which a shielded 100-watt lamp was set. The test plate was level with the bottom of the slit and rested on a dull black surface. A little light was diffusely reflected up from the surface of the test plate but the greater part of the light reached the insect directly from the horizontal beam. The light intensities at the two ends of the test plate were 1.8 and 0.6 f.c. respectively.[2] The path taken by the insect was followed with a pencil, and marked directly on the plate. This did not appear to disturb the beetle.

Light adaptation was effected by placing the beetle, in water in a glass cell ($2\frac{3}{4}'' \times \frac{3}{4}''$) for 20 minutes, in the path of a beam of light from a 500-watt projection lamp. A strongly-reflecting surface at the "darker end" of the cell ensured fairly uniform lighting. Intensity of adapting light was 4,800 f.c.[2] Heating was avoided during the light adaptation period by interposing heat-absorbing filters.

At the beginning of each experiment, the beetle was placed by means of a piece of stiff paper at approximately the centre of the test plate. It was not headed precisely in any special direction, as earlier experiments had shown that the animal oriented immediately, irrespective of the direction in which it was placed.

Observations

With all eyes functional, a strong, positively phototropic response was invariably obtained, whether the animal was light- or dark-adapted.

When any two, or when only one of the four eyes[3] was functional, a negative response was obtained if the beetle were dark-adapted. If only one eye was blackened the animal was photopositive. In the great majority of experiments, either the two upper, or the two lower eyes were covered. This avoided any complications, such as possible circus movements (cf. Clark).

It was often impossible to carry out all the experiments, with the various combinations of eyes, on the same individual, but the behavior was sufficiently constant to combine the results obtained from different animals. (A very few individuals were found in which the phototropic reactions were irregular.)

[2] Light intensities were measured with a Macbeth Illuminometer, using dense filters for the highest intensities.

[3] To obviate needless repetition, the upper and lower halves of the eyes are simply referred to in the results as upper and lower eyes.

The results of experiments with (*a*) two upper eyes only functional and (*b*) two lower eyes only functional were similar. The beetles were always positively phototropic when they were light-adapted. They were usually still positively phototropic after dark adaptation of less than two hours duration, but became negatively phototropic after dark adap-

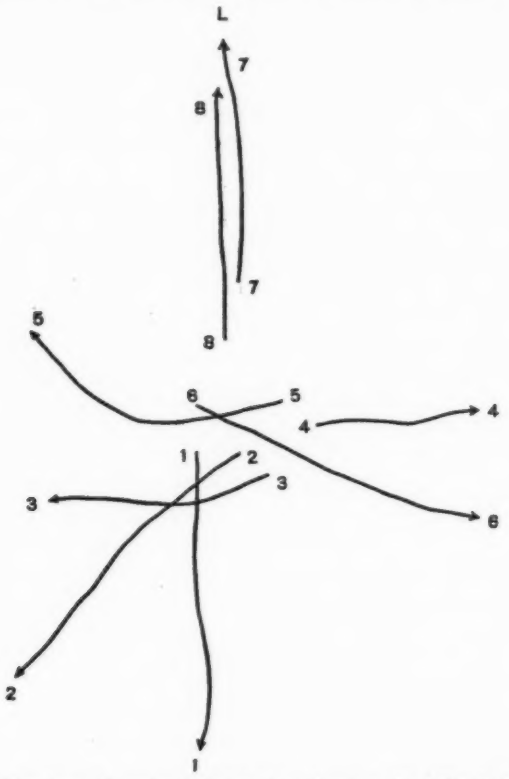

FIG. 1. Tracings of paths followed by *Dineutes* (Individual *X*) May 19, 1938, in 8 successive trials after dark adaptation for seven hours. The upper eyes were functional. The numbers refer to the order of trials. *L.* indicates light source.

tation of more than two hours duration. However, the actual period of dark adaptation necessary to bring about the change in phototropic sign showed considerable individual variation; in a very few cases, even one hour was sufficient.

A beetle which had been dark-adapted for a period sufficient to be-

come negatively phototropic would show a reversal to the original positive condition as it became partially light-adapted. This was shown as follows. A beetle, after dark adaptation for many hours, was tested repeatedly in the beam. The responses exhibited at first were all photonegative, but the sign of phototropism became reversed after a certain

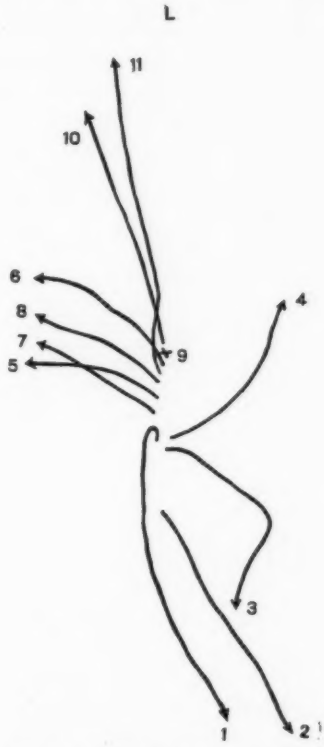

FIG. 2. Tracings of paths followed by *Dineutes* (Individual *B*) December 15, 1937, in 11 successive trials after dark adaptation for twelve hours. The upper eyes were functional. No "run" was obtained in Trial 9.

amount of light adaptation had been brought about by the light from the test beam itself. Subsequently, all further responses were consistently photopositive.

This reversal of phototropic sign with light adaptation was also observed, while avoiding the repetition of trials. A fully dark-adapted beetle was tested once to demonstrate the negatively phototropic reaction.

It was then left in the test beam, surrounded by a small glass cell, until the light effected a sufficient degree of light adaptation. A single new trial then showed the beetle to be positively phototropic.

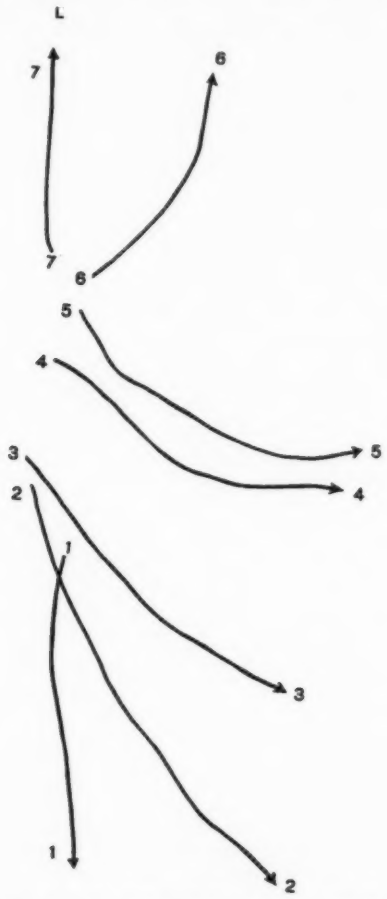

FIG. 3. Tracings of paths followed by *Dineutes* (Individual *V*) May 20, 1938, in 7 successive trials after dark adaptation for seven hours. The lower eyes were functional.

It was obvious that a considerable period of exposure to darkness was necessary to elicit the negatively phototropic reaction. Experiments were next carried out to test the possibility that with all eyes functional,

a very prolonged period in darkness might cause a reversal of phototropic sign. The results of these experiments, with seven individuals, showed that reversal cannot be brought about by 24–65 hours of dark adaptation, if all eyes are functional.

If the two upper or the two lower eyes were blackened but a very few ommatidia of one of these eyes were left exposed, the beetle remained photopositive when fully dark-adapted. On completely covering the eye, the beetle, when dark-adapted, became negatively phototropic.

A few experiments were carried out with any three eyes blackened, and other experiments in which one upper eye and one lower eye of the opposite side were blackened. Such experiments, with any two or any three eyes covered, always showed that the dark-adapted beetles were negatively phototropic, and that light adaptation caused a reversal to positive phototropism.

TABLE I

Change in the direction of phototropic path followed by Dineutes (Individual V), in 7 successive trials, consequent upon light adaptation. The animal was first dark-adapted for 7 hours. The lower eyes were functional.

Trial Number	Angle of Deviation from Normal Positive Path
	degrees
1	180
2	150
3	135
4	110
5	110
6	50
7	0

Exact time of each trial, since beginning of exposure to test light was not recorded.

On the orthodox Loebian view, it would be expected that on repeatedly testing an originally fully dark-adapted beetle, a number of photonegative trials would be first obtained, and then, if a reversal of sign occurs with light adaptation, a sudden and complete change to positive phototropism would be observable. It was actually found, however, that an incompletely light-adapted beetle pursued a path *at an angle* to the light beam. If the direct negative response may be regarded as a deviation of 180° from the positive path, then with exposure to light, the angle of deviation gradually diminished until it approached 0° (i.e. the beetle was again positively phototropic).

It was possible to obtain such records from several individuals, and to repeat the observations on the same beetle. Although the actual paths (Figs. 1–3) were not straight, and in spite of some irregularities

TABLE II

Change in the direction of phototropic path followed by Dineutes (Individual B), in 6 successive trials, consequent upon light adaptation. The animal was first dark-adapted for 12 hours. The upper eyes were functional.

Trial Number	Total Time of Exposure to Test Beam	Angle of Deviation from Normal Positive Path
	minutes	*degrees*
1	0	180
2	1	120
3	3	75
4	8	60
5	18	(no "run" obtained)
6	38	20

in respect of angle, in general the results showed a surprisingly consistent trend.

The time required for complete reversal of phototropic sign varied from < 10 to > 30 minutes. It is believed that a rough estimate of

TABLE III

The effect of dark adaptation on the direction of phototropic path in Dineutes (Individual V). The lower eyes were functional.

Time in Dark Following Standard Light Adaptation	Result of Single Trial in Test Beam: Angle of Deviation from Normal Positive Path
minutes	*degrees*
5	5
15	35
24	90
30	(140)
45	120
90	160
180	180

the course of light adaptation can be obtained by measuring the successive deviation angles (Table I).

Table II shows an experiment conducted on a beetle which was dark-adapted for a longer period. The total duration of exposure to light from the test beam since the beginning of the experiment was also recorded for each trial.

If these results do really indicate the course of light adaptation, it should be possible, using similar methods, to follow the course of dark adaptation. To test this possibility, a number of experiments were carried out using different periods of dark adaptation. In each experiment the animal was first light-adapted by means of the usual adapting light for 20 minutes. It was then dark-adapted for a given period, and a single trial made in the test beam. By using various periods of dark

adaptation a number of trials were obtained, and the results (e.g. Table III) showed that with progressively longer periods of dark adaptation, the paths pursued showed an increasingly greater deviation from the positive path. An approximate dark-adaptation curve has been constructed from these data (Fig. 4) for one individual.

The possibility that the change in the paths pursued might be caused by the repeated disturbance of the beetle when replacing it at the centre of the test plate was investigated. The test plate was constantly moved in such a way that a beetle which had been originally fully dark-adapted was kept in the beam without being replaced. The animal at first

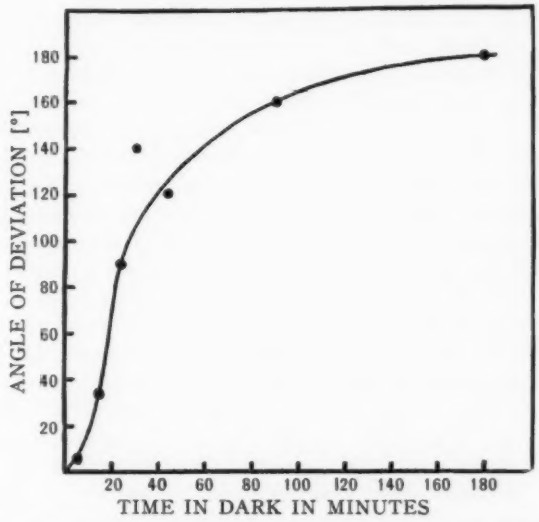

FIG. 4. Progressive deviation from the normal positively phototropic path during reversal of phototropic sign with dark adaptation, in *Dineutes horni* (Individual *V*). (The lower eyes were functional.)

moved from the light source, but with continued exposure it turned gradually towards the source until it reached the edge of the test plate. The changes in path are therefore not the result of disturbance, but do depend on the degree of adaptation.

DISCUSSION

McIndoo (1929) states that Schmitt-Auracher believed there was a relationship between the state of adaptation and pigment deposition in insect ocelli and the sign of phototaxis. In the present observations

on *Dineutes,* a reversal of phototropic sign could never be obtained when all four eyes were functional, but if *any* two, or *any* three, eyes were blackened, the fully dark-adapted beetles were always negatively phototropic. The difference in behavior may depend upon the area of photosensitive surface stimulated. Although one cannot compare human, subjective sensations with animal tropisms, it is interesting that experiments on the intensity discrimination of the human eye have shown that the use of a small test field of high intensity may cause uncomfortable glare and even pain, while with a larger test field of the same average intensity, vision is normal.

When only partially light- (or dark-) adapted, *Dineutes,* with only two eyes functional, moves at an angle to the light beam. Rádl, Carpenter, and especially Dolley (1916) and Clark (1931 and 1933) have discussed movement of phototropic insects at an angle to a beam of light, but in all such cases only *one* of a pair of symmetrical eyes was functional, and deviation might be then expected. Clark supposed that light from the direct beam, and light from the background acted on the functional eye. But in the experiments described, with both upper, or both lower eyes functional, light from both background and beam should act equally on the two sides, and therefore, according to Loeb, the insect should move *directly* to or from the source. Indeed, Clark states that if in *Dineutes* both upper or both lower eyes are blackened, the beetle moves straight towards the light. But in Clark's experiments the beetles were consistently photopositive, and provided *D. horni* is strongly positively phototropic, it moves straight to the source also.

Mast (1938) has shown that the phototropic reflexes in insects vary according to the region of the eye stimulated. In *Dineutes,* according to Clark, the posterior ommatidia are much more sensitive than the anterior ommatidia. Possibly then, during reversal of phototropic sign with light adaptation in *D. horni,* some of the ommatidia become light-adapted more rapidly and give rise to reflexes which are opposed by the less sensitive ommatidia. Morphologically symmetrical ommatidia also may not adapt at exactly similar rates, and therefore photochemical reactions will proceed at different rates on the two sides during partial adaptation, and a deviation would result. When *all* ommatidia are fully adapted, the beetle will move straight to the source.[4]

Although an exact explanation must therefore await further work, it is obvious that the simple Loebian theory will not account for the facts here presented. Light must act in a more complex manner, and among

[4] Some recent experiments on the related genus, *Gyrinus,* have shown that even with all eyes functional, a positively phototropic beetle, when it is fully dark-adapted, may pursue a path deviating widely from the normal straight photopositive path.

other factors, the phototropic responses must depend to a considerable extent upon the region of eye stimulated, as Mast has repeatedly emphasized.

Summary

1. When all eyes are functional, *Dineutes horni* is positively phototropic after dark or light adaptation.

2. With one or with two eyes functional, *Dineutes* is positively phototropic when light-adapted, but is negatively phototropic when fully dark-adapted.

3. At intermediate stages of dark and light adaptation, the beetle moves at an angle to the light rays. The courses of dark and of light adaptation were followed by a study of these "angles of deviation" from the incident rays.

4. Possible theories are discussed to account for these results.

ACKNOWLEDGMENTS

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MODIFIED SEXUAL PHOTOPERIODICITY IN COTTONTAIL RABBITS[1]

THOMAS HUME BISSONNETTE AND ALBERT GEORGE CSECH

(From the Shade Swamp Sanctuary, Farmington, Connecticut)

Introduction

Studies too numerous to cite here show that the sexual cycles of many birds and mammals and of some fish and reptiles can be modified and their breeding seasons changed by manipulating daily cycles of exposure to light. Not all are susceptible in the same way nor to the same degree (Bissonnette, 1936, 1938; Marshall, 1936, 1937).

The development of proper methods of conservation and wild life management require knowledge as to what wild animals have photoperiodic breeding cycles and how they react to management of light-cycles. Breeding seasons of some animals have been prolonged to permit two litters per year in place of one, with better than normal growth of early-induced litters (Bissonnette and Csech, 1937, 1938, 1939).

The cotton-tail or gray rabbit of New England (*Sylvilagas transitionalis* (Bangs)) is shot for sport and food, furnishes food for fur-bearing carnivores, and may injure fruit trees in some places and seasons. It exhibits a limited breeding season with three or four litters, beginning about mid-April. It is, therefore, more polyoestrous than most of the animals so far investigated and intermediate between strictly monoestrous or dioestrous and completely polyoestrous forms. It, therefore, has been tested by "night-lighting" in autumn and winter.

Material and Methods

Three pairs of cotton-tails were placed, each in a wire enclosure raised from the ground, with wooden "den" at one end. In each den a few inches of earth were covered with loose dry grass about two inches deep for bedding. Twenty-five-watt bulbs were so placed as to shine into both den and enclosure and controlled by a time switch so that lights were lit for one hour each night for the first week, beginning on October 10. "Night-lighting" was increased one hour each

[1] Aided by grants from the National Research Council, Committee for Research in Problems of Sex, 1935–8, and from the Penrose Fund, American Philosophical Society, 1938–9, and by coöperation and animals from the State Department of Fish and Game, Connecticut.

ten days thereafter to eight hours on December 17 and maintained into February, and, in one case, into April. Lights came on each evening at six o'clock. All pens were outside, without heating, except from the bulb, throughout the experiment. Feeding and care were similar for all rabbits at the sanctuary.

Replacements were made without altering schedule when animals killed one another and the exact lighting history of each animal recorded. The gonads of killed animals were secured for histological study. But none were sacrificed expressly for such material, because our experience with raccoons and the behavior of these rabbits suggested that matings would lead to litters out of season. Sex-organs were obtained also from unlighted males on December 8 and January 25 for comparison with those of a male killed by his mate, January 12, after night-lighting eight hours each night from December 20.

Observations and Results

After varying periods of lighting and after matings, two experimental females killed their original mates by biting them through the back. One male killed his mate by grasping her anal region with his teeth and pulling out her abdominal organs which became useless for study. Replacement of males may account for failure of matings to induce pregnancy in December and again beginning on January 5. Controls also mated somewhat later in December and about January 10, all without pregnancies. Bissonnette's studies on male ferrets indicate that willingness to mate antedates potency and fertility by a considerable time. This was probably true here also.

After matings in January, two experimental females made nests and one lined hers with fur to receive young that failed to come. No control did so then, nor until near littering time in April.

The first "experimental" litter (of two) was born on April 4 and died April 10, from exposure. The nest was not heated and the mother left it for long periods. On May 31 and June 30 she produced second and third litters (of one and six) which survived. The first "control" litter came on April 18 and all six died of exposure. None of the other experimental or control females had litters before June 8, although the above-mentioned control female mated again the day her litter was born and others probably did so too. In that season no controls had litters live through. They normally should have had litters every thirty days after mid-April. It was a poor rabbit-breeding season, for reasons as yet unknown. Experimentally lighted animals succeeded slightly better than controls on normal light.

A male, used for replacement on December 20, lighted eight hours

each night until killed by his mate on January 12, had mated about January 5–7 with her. His testes showed sperms just metamorphosed in small numbers in some tubules but none had yet reached the middle part of the epididymis. The apparent breeding condition of this epididymis, with tall columnar epithelium and well developed ciliary processes, indicates functional activity of the interstitial cells of the testis and accounts for sexual libido and matings.

Much smaller testes from the control of December 8 showed smaller tubules with no stages of germ-cells beyond synizesis. Its epididymis, in a partly activated, partly regressed condition, suggested some activity of interstitial cells, which may account for December and January matings of controls. Its epithelium was short columnar with some ciliary processes, less developed than those of the lighted male.

Testes from the January twenty-fifth control were slightly more advanced in spermatogenesis than those of December 8. Its most advanced stages were synizesis and a few growing primary spermatocytes. No germ-cell debris was found in the middle part of the epididymis which was more regressed than that of December 8. Its lining epithelium was more nearly cuboidal, with no ciliary processes evident.

Conclusions

Increasing night-lighting induces cotton-tail rabbits to undergo sexual activation in winter. In males, it leads to complete libido and spermatogenesis in twenty-three days at January 12, on eight hours of added light from December 20. It would probably induce complete breeding effectiveness in little longer time. In females, it induces repeated receptivity followed by nest-making and even lining of nests with fur.

December and January matings do not alone indicate increased sexual activation, for controls on normal light reacted similarly. Killing of mates is not attributable to added light; but rather it was permitted by the close confinement's preventing escape from an aggressor. Making and lining of nests in January, not done by controls at that time, signify activation above the normal for that time, and suggest pseudo-pregnancy after winter copulations.

Even if litters can be born under the conditions of temperature prevailing in these experiments in winter, they cannot be raised by their mothers, even in their fur-lined nests. The mothers do not keep the nests warm, as do raccoons, but leave them closed for long periods, returning only at intervals to suckle the young, born naked and defenseless against cold. It is suggested, however, that, with warmed nesting places, the long absences of the mother may not permit the young to die nor prevent the raising of litters in winter. In addition, by arranging run-

ways so that males and females can have separate dens and can be separated after matings by a wire screen which permits them to remain acquainted without being able to kill each other, the same pairs may be kept on the lighting schedule and make winter breeding successful. Further experiments along these lines seem to be indicated.

Summary

1. Three pairs of cotton-tail rabbits were confined in dens and runways and subjected to increased lighting at night from October 10 onward. Controls were not lighted but fed similarly.

2. The original objective, induction of winter litters, was not attained because, after varying times of lighting, one member of the pair killed the other and replacements were made on schedule.

3. Sex organs of males were modified to complete sperm formation in twenty-three days in December and January and mating libido reached, accompanied by breeding conditions of the epididymis. Controls showed mating libido but no spermatogenesis nor epididymal activation.

4. Lighted females mated and made nests; and one lined its nest with fur. Controls mated but made no nests. No pregnancies resulted with any female until April.

5. The induced changes indicate that these rabbits can be brought into breeding condition in winter by increased lighting, but modification of the method used and the provision of warmed nest-boxes are necessary for successful winter breeding and rearing of these animals.

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THE LIFE CYCLE OF DACTYLOMETRA QUINQUECIRRHA, L. AGASSIZ IN THE CHESAPEAKE BAY[1]

ROBERT A. LITTLEFORD

(From the Chesapeake Biological Laboratory, Solomon's Island, Maryland)

Introduction

The common jellyfish or sea nettle of the Chesapeake Bay is the large scyphozoan medusa *Dactylometra quinquecirrha*, L. Agassiz. This species is a coastal form occurring in tropical and temperate seas throughout the world. In the Chesapeake Bay, as in other brackish water areas (Mayer, 1910; Menon, 1930), it is accompanied by a colorless, milky-white medusa which lacks the pigmented areas of *Dactylometra.*

The primary object of this study has been to elucidate the problems presented by the white medusa, which becomes sexually mature in a growth stage having twenty-four marginal tentacles, thus answering the taxonomic description of the genus *Chrysaora* of Eschscholtz. Certain workers, including Bigelow (1880), have considered the white medusa a member of the genus *Chrysaora,* on a basis of the sexually mature form, possessing twenty-four marginal tentacles. Mayer (1910) considers this medusa to be a growth stage of *Dactylometra quinquecirrha,* the so-called "Chrysaora-stage," believing that the premature development of the gonads is the result of the brackish water conditions. More recently Papenfuss (1936) has considered this white medusa to be a variety of *D. quinquecirrha* and named it *chesapeakeii.*

The literature on the life cycles of these two forms is limited to brief and scattered observations by Mayer (1910), Stiasny (1919–1921), Papenfuss (1934), and Truitt (1934). For this reason an investigation of the complete life cycles of the red and the white medusae was undertaken. The results obtained from this investigation are presented at this time.

Procedure

The organism was reared from the egg stage to the medusa under controlled conditions, and the data obtained in this way were supple-

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mented by observations in nature. Fertilization was obtained in the laboratory (1) by crowding sexually mature males and females together in a small container, and (2) by placing segments of the ripe gonads in fingerbowls 4 by 10 cm. in size and partly filled with brackish water.

The data reported in this paper are based on observations on between two and three thousand individual scyphostomae, about five hundred of which have been reared through four successive years. About one thousand additional embryos were followed to the scyphostoma stage.

The embryonic material was reared in a basement room of the laboratory, where changes in temperature and light were comparatively slight, though not controlled. The temperature of the water in the fingerbowls varied between 20 and 24° C. The lighting was indirect, light entering the room through a small window just below the ceiling on the eastern wall.

Scyphostomae were reared in the laboratory by placing a fingerbowl in a battery jar of three-liter capacity partly filled with bay water. Others were reared in fingerbowls placed overboard under natural conditions in nearby waters and protected by wire cages measuring 24 × 9 × 9″. The wire was of hardware screen measuring eight strands to the inch. Each cage, containing five bowls held in a wooden rack, was lowered to the bottom in eight or ten feet of water in a protected cove near the laboratory.

The water containing the free developing embryos was changed daily by pipetting out a part of it and refilling the jars with fresh brackish water, whereas the water on the attached forms was changed weekly. The larvae were fed daily on material gathered by means of plankton tows, and on small pieces of oyster.

The embryonic material was studied by using hanging drops and depression slides, which made it possible to follow development of a single egg or group of eggs up to and through the planula stage. Material intended for cytological or histological examination was fixed in standard solutions of Kaiser's., Bouin's, Flemming's, or Zenker's fixatives and stained according to standard methods. Iron hematoxylin and Mallory's triple stain as given by Galigher proved to be particularly effective. Chloral-hydrate-menthol was most satisfactory for anesthetizing the scyphostomae.

Observations

Mature males and females of *D. quinquecirrha* may be recognized on a basis of the color of the gonads, which in the males are bright pink

and in the females grayish brown or yellowish brown in appearance. The egg is a colorless cell with a prominent nucleus. The mature egg (Fig. 2), which is yellow in color, resulting from the filling of the cytoplasm with yolk material, varies in diameter from 0.07 to 0.19 mm., with an average diameter of 0.15 mm. The spermatozoa are developed in sacs (Fig. 1), many of which are found in a single gonad. These sacs show great variation in size and shape.

The eggs are released from the gonads into the stomach cavity and fertilization takes place either there or externally. Numerous laboratory observations strongly suggest that fertilization occurs in the stomach cavity. This is substantiated by the collection of fifty-one females bearing embryos in the gastric cavity.

The relationship between the initiation of development and the time of day is shown in Table I, which is based on 710 cases. These results are supported (1) by the collection, between eight and nine o'clock in the evening, of females having eggs in the first and second cleavage stages, and (2) by the collection before ten o'clock the next morning of females bearing gastrula and planula stages in the gastric cavity.

TABLE I

Time of Fertilization

Time	Number	Time	Number
3:00–6:00 p.m.	0	9:00–10:00 p.m.	206
6:00–7:00 p.m.	2	10:00–11:00 p.m.	24
7:00–8:00 p.m.	124	11:00–12:00 p.m.	16
8:00–9:00 p.m.	329	after 12:00 p.m.	9

The fertilized egg (Fig 3), which is distinguished by the presence of a distinct membrane, may divide immediately or it may remain quiescent for six or seven hours after fertilization. The first sign of development is the elongation of one end of the egg (Fig. 4), producing a prominent, knob-like protrusion (Fig. 5). This knob, the function of which is unknown, is resorbed prior to the first cleavage, which oc-

EXPLANATION OF PLATE I

Photomicrographs from living material.

FIG. 1. Section of gonad of male medusa showing sacs containing spermatozoa. × 146.

FIG. 2. Section of gonad of female medusa showing mature and immature eggs. × 146.

FIG. 3. The fertilized egg. × 182.

FIG. 4. Elongation of the fertilized egg. × 182.

FIG. 5. Knob-like protrusion of fertilized egg. × 95.

FIG. 6. The two-celled embryo. × 146.

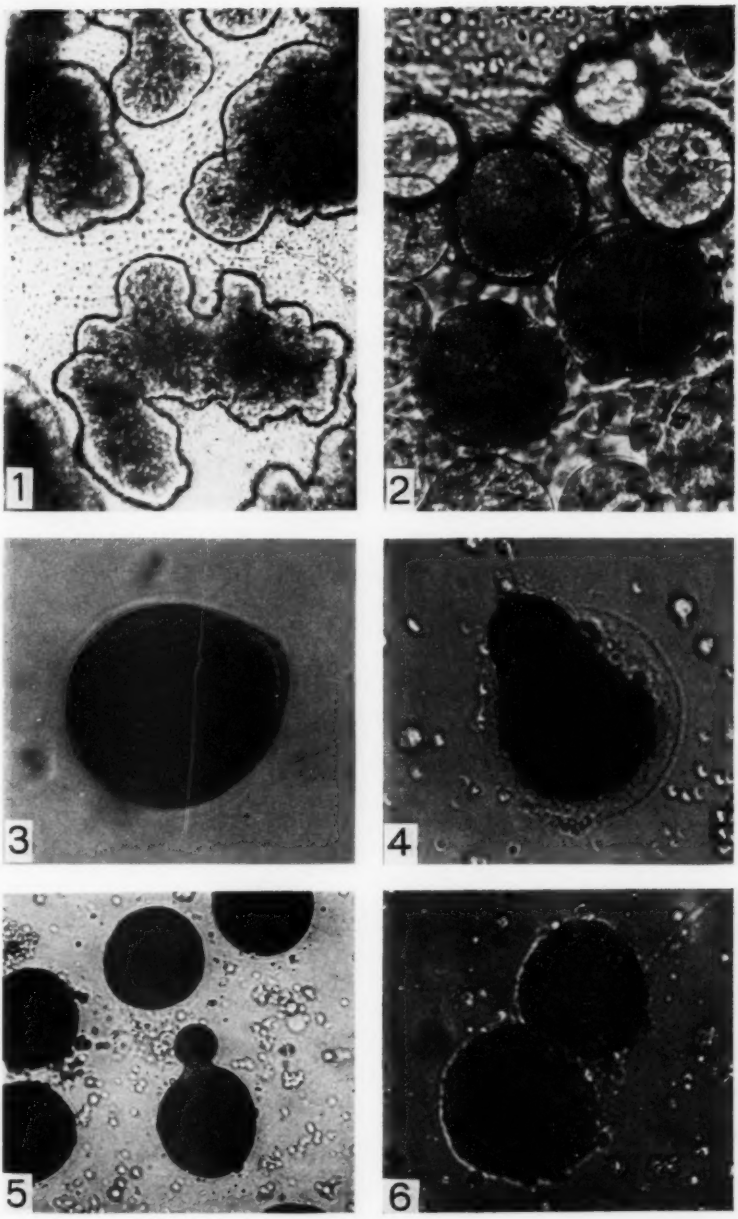


PLATE I

curs a little less than one hour after fertilization. The two-celled embryo (Fig 6) divides almost immediately to form four equal blastomeres (Fig. 7). At the end of three or four hours, under laboratory conditions, a third cleavage results in an embryo of eight equal cells. Succeeding divisions result in the formation of a blastula in about ten or twelve hours. During these cleavage stages the fertilization membrane, which surrounded the egg and embryo during early segmentation, is lost. The cells on one end of the blastula now invaginate and a free-swimming planula develops within sixteen to twenty hours after fertilization.

No provision is made for the protection of the developing embryo, such as the pockets on the oral arms of *Aurellia*. The rate of development of the embryonic stages shows great variation among individuals as well as with the external factors of the environment. A more detailed discussion of the stages of cleavage and gastrulation is to be presented in a subsequent paper.

The planula (Fig. 8) is at first round or oval in shape, but within two or three hours it adopts a definite pyriform outline. The first sign of movement in the planula is a regular rotation that is observed at the end of twenty or twenty-four hours. This is produced by the action of cilia which are developed by certain cells of the ectoderm. After adopting the pyriform outline, the planula moves through the water by means of a fringe of cilia confined to the cells of the periphery. Movement is rapid and the direction is changed continuously. The broad end is always directed anteriorly.

The Scyphostoma

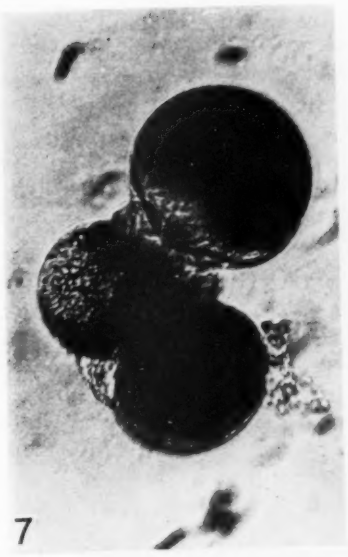
Completing its free-swimming existence after a period varying from three to five days, the planula becomes attached to some object, and is then known as the scyphostoma or hydra-tuba. The larva thus formed has a "ninepin" shape. In the process of attachment, the cells of the anterior region secrete an adhesive substance which forms a disc surrounding the stalk of the polyp.

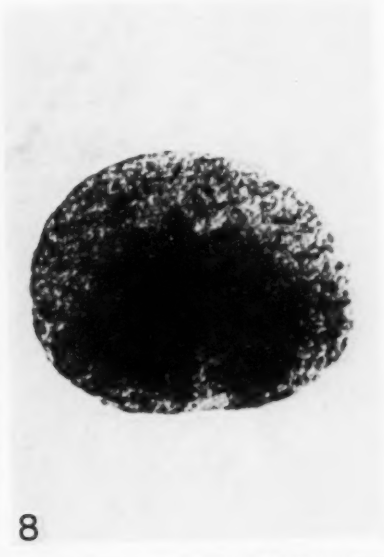
The scyphostoma stage of *D. quinquecirrha* follows the general pattern of the group and lends support to the statement of Fowler (1900)

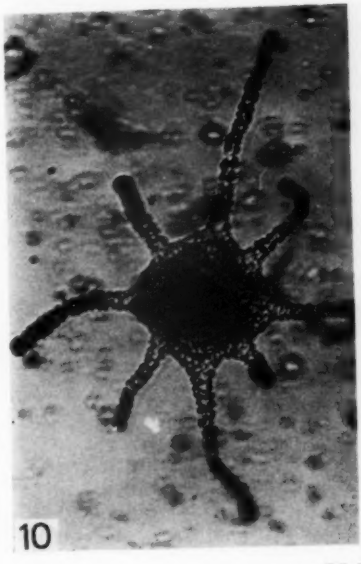
EXPLANATION OF PLATE II

Photomicrographs from living material.

Fig. 7. The four-celled embryo. × 165.
Fig. 8. The planula previous to adopting a pyriform outline. × 165.
Fig. 9. Scyphostomae having three to seven tentacles. × 26.
Fig. 10. Schyphostoma having eight tentacles. × 66.







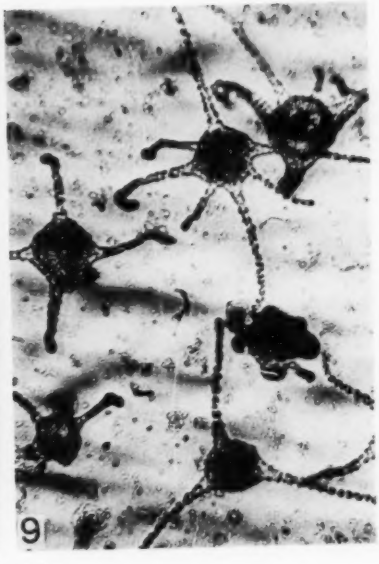


PLATE II

that the scyphozoan polyp is insignificant in size and monotonous in structure. The adult scyphostoma, which has sixteen or, rarely, twenty tentacles, shows three clearly defined stages of intermediate development, having four, eight, and twelve tentacles respectively. This fact probably explains the observations of Mayer (1910) that reproduction was through a scyphostoma stage having normally four tentacles, and of Stiasny (1919) that the number of tentacles was eight.

Development

Immediately after attachment, the oral region shows a great proliferation of endodermal cells resulting in the formation of the oral cone. This development, in turn, stretches the ectodermal layer and changes the mouth from a small almost invisible slit into a wide, yawning opening. This process is completed in from one to three hours after attachment. The sides of the oral cone become secondarily cleft to form a cruciform mouth.

The tentacles are produced singly and arise as wart-like evaginations from the region of the body surrounding the mouth. At the end of five days, the scyphostoma has four primary tentacles (Fig. 9) developed in the perradii of the body. Alternating with these tentacles, there arise four taeniolae, or ridges of the ectoderm, projecting into the stomach cavity in the interradial axes.

A circular depression appears on the upper part of the larva on the sixth day, marking off the oral cone from the bases of the tentacles. Several days later four deep depressions, the septal funnels, appear in this furrow just above the taeniolae. At the end of ten days a scyphostoma with eight tentacles (Fig. 10) is produced as the result of the formation of four secondary tentacles in the four remaining interradii. The scyphostoma continues to increase in size, and at the end of fifteen days, eight more tentacles have been developed in the adradii of the polyp (Fig. 11). Although the typical number of tentacles is sixteen, occasionally an individual which has developed twenty tentacles will be noted. This, however, is a rare exception.

EXPLANATION OF PLATE III

Photomicrographs from living material.

FIG. 11. Oral view of scyphostoma having sixteen tentacles. × 26.
FIG. 12. Lateral view of scyphostoma showing the pedal discs. × 26.
FIG. 13. Colony of scyphostomae. × 10.
FIG. 14. Apical view of the strobila. × 26.
FIG. 15. The ephyra immediately after being released. × 46.
FIG. 16. Contracting ephyra showing the cruciform mouth, the heavy nematocyst aggregations, and the beginning of the gastric cirrha. × 119.

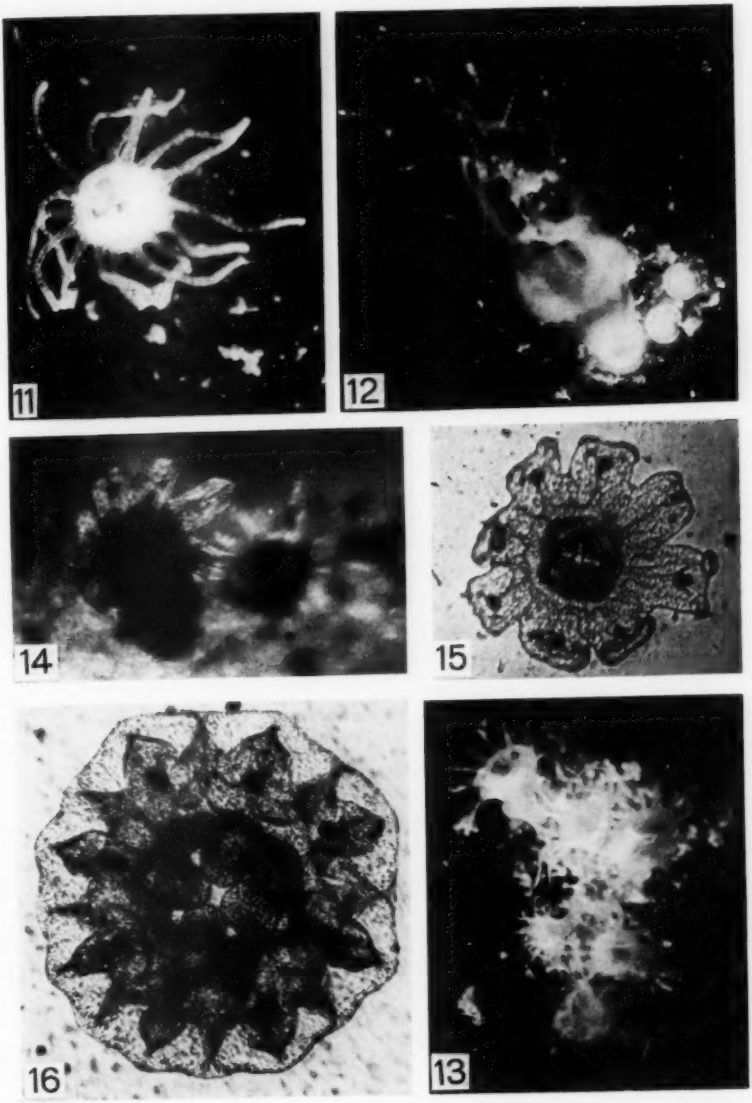


PLATE III

In typical specimens, the scyphostoma has reached complete development by the middle of August, although longer periods are required for certain individuals. The only observed change taking place from September until the following April or May is a slight increase in size. The scyphostoma then becomes bright pink in color and begins to undergo a process of transverse fission known as strobilization.

After strobilization is completed, the remaining scyphostoma, which is about one millimeter in height, has a small cruciform oral slit, surrounded by sixteen normal-size tentacles produced before the ephyral discs were released. It may be distinguished from those scyphostomae which have not undergone strobilization by (1) the small mouth opening, (2) the short and broad appearance of the body, and (3) the crater-like mouth area in contrast to the normal oral cone. This old scyphostoma regains its normal appearance in from five to seven days. The following spring it again undergoes strobilization, producing the typical number of ephyrae.

Associated groups or colonies of scyphostomae (Fig. 13) are a regular occurrence in *D. quinquecirrha*. Such colonies, consisting of seven or eight individuals ranging in height from one to four millimeters, are produced by means of (1) stolons, (2) buds from the stalk of the parent scyphostoma, and (3) development of scyphostomae from the pedal discs. The pedal discs (Fig. 12) are formed as a result of movement of the scyphostoma from place to place, in the course of which it leaves behind on the substrate a protoplasmic disc. The groups of polyps developed from these discs may be either linear or irregular in formation, depending on the movement of the "parent" scyphostoma. The colonies formed either by means of buds from the stalk or by means of stolons are irregular in arrangement because of the irregular development of buds and stolons.

During the course of this study, it was observed that certain culture dishes in open water no longer contained scyphostomae. Instead, the bottom surface was covered with small, brownish, wart-like cysts. In one case (December, 1935) 51 cysts were counted and their positions marked on the culture dishes. The following February, 21 cysts had disintegrated, while normal scyphostomae had developed in the remaining 30 cases. In November, 1937, several culture dishes containing cysts were brought into the laboratory. The cysts in these dishes were kept under constant observation from that time. A count of 27 cysts was made in one dish, and two weeks later it was observed that the number was reduced to eighteen. Scyphostomae were produced from these cysts, and development took place in a normal manner.

It is to be noted that the cysts occurred in both cases in material

that was being held in cages under conditions as nearly natural as possible. Scyphostomae which were retained in the laboratory for long periods without food or change in water resorbed themselves until they diminished to the size of one-week-old forms, but at no time did they encyst. At present, therefore, we can offer no valid explanation for the production of cysts. The same phenomenon has been observed in *Chrysaora* by Chuin (1930), who also has been unable to offer an adequate explanation. In *Chrysaora* these cysts produce a ciliated larva which swims about and then attaches itself to develop into a polyp. In *D. quinquecirrha* a larva was not produced, but a large number of these cysts produced new polyps in the same manner as has been reported for pedal discs.

Morphology

The scyphostoma of *D. quinquecirrha* is a colorless, goblet-shaped polyp averaging 3.5 mm. in height, with a diameter of 0.6 mm. The mouth is cruciform and occupies the entire oral surface of the organism. The body of the scyphostoma is divided into two distinct regions: (1) the long, stalk-like, tapering basal region, and (2) the cup-like body, or apical region. The relative size of the two parts is dependent upon the degree of contraction of the polyp. The tentacles are solid, averaging 6 mm. in length, and bear many nematocysts, which are regularly arranged, forming a series of successive rings around the tentacle. The aboral end of the stalk is fixed to a sub-strate and is surrounded at the point of attachment by a pedal disc. In older specimens the pedal disc may be one of a group, each of which marks a previous place of attachment. The body wall is divided into the three layers, ectoderm, endoderm, and mesoglea. The mesoglea is a thin, almost invisible lamella separating the two germ layers in the early development of the scyphostoma. This gelatinous layer increases after the eight-tentacle stage until it becomes a prominent structure of the mature polyp, containing muscle fibers and other cellular structures produced by the germ layers.

Reproduction

Reproduction in the scyphostoma stage is of two types: (1) budding, resulting in the production of additional polyps, and (2) transverse fission, or strobilization, resulting in the production of ephyrae.

Additional scyphostomae are produced as the result of three types of budding: (1) somatic buds from the stalk region of the polyp, (2) production of stolons, which may be considered a form of somatic budding, and (3) development from pedal discs.

Stolon formation and somatic budding, the common methods of asexual reproduction in the polyp stage of the *Scyphomedusae,* have been found to be rare in this species. Likewise the statement of Truitt (1934), that the polyp buds profusely, has not been borne out by this study. The common method of asexual budding in *D. quinquecirrha* was found to be by means of pedal discs, the formation of which has been reported by Hérouard (1907) as occurring in *Taeniolhydra roscoffensis.* Other investigators (Mayer, 1910) have claimed that Hérouard was dealing with an abnormality of development of *Aurellia.* This study has shown, however, that the formation of pedal discs is a normal occurrence in *D. quinquecirrha.*

Strobilization occurs in April or early May (June or July under controlled conditions). At this time the scyphostomae become bright pink in color and develop a series of circular furrows in the wall of the flask-like part of the body. As these furrows become deeper, the oral tentacles of the polyp are resorbed. This process may be completed before further development of the furrows occurs, or the tentacles may remain until the furrows mark off a series of well-defined discs. The furrows eventually divide the polyp into a series of saucer-like segments connected by a central cord and borne on a slender stem. Each of these saucers develops eight deep clefts, which in turn separate the periphery into eight lobes. Each lobe becomes cleft to form a bifurcation. At the apex of each bifurcation a deeply pigmented club, the future tentaculocyst of the medusa, is produced. The scyphostoma reaches the stage of a strobila (Fig. 14) in a period of four or five days and the saucers are then released into the water.

The number of discs produced remains remarkably constant at either five or six. The process of separation of the discs from the base or stalk region of the scyphostoma is completed in from ten to sixteen hours. Shortly before being released, the discs begin to pulsate and continue a characteristic movement of short, rapid pulsations until release is gained.

The Ephyra

The general structure of the ephyra was observed by Stiasny (1921), and Mayer (1910) had earlier published figures of the ephyra drawn by Brooks.

The newly liberated ephyra (Fig. 15) is about 0.84 mm. in diameter, measured from the distal end of one arm to the distal end of the opposite arm. The most prominent structure of its anatomy is the rhophalia, or tentaculocyst, of which there are eight, one in the bifurcation of each of the ephyral arms. Tentacles are lacking. The manubrium measures

approximately 0.23 mm. in length and is flared in the radii, forming a cruciform structure. The nematocysts are grouped in capsules of three different sizes and also appear singly covering the exumbrellar surface. The ends of the ephyral arms appear knobbed as a result of nematocyst aggregations. The middle of each of the arms is marked by the presence of a pair of large nematocyst capsules, and the region where the arms join the disc is heavily covered with nematocysts.

The ephyra spends the first three or four days of its existence close to the bottom. During this time the subumbrellar surface is outward and the manubrium is carried in an upright position. A single tentacle is developed in each of the deep clefts that separate the ephyral arms. These tentacles, which appear four or five days after the ephyra has become separated from the scyphostoma, are the eight primary tentacles of the medusa, and the lobes of the arms are the primary lappets of the bell margin. Following the appearance of the eight primary tentacles, the ephyral disc grows outward, filling the clefts which separate the arms until it reaches the radius of the rhophalia. The margin then becomes cleft into a series of lappets, the number of which varies with the age, bell diameter, and stage of development of the individual.

When the eight tentacles have formed and the manubrium has increased in length until it is slightly longer than the bell diameter, the bell becomes inverted and the manubrium hangs downward from the center of the subumbrella. Inversion occurs between the sixth and the eighth day in typical specimens. The oral lips of the ephyra, which are heavily covered with nematocysts, are simple folds of the body wall, produced from the connecting tube of the strobila. As the ephyra develops into the medusa, these lips become folded and develop curtain-like margins on their internal edges.

Four hollow, tentacle-like outgrowths, which are the first gastric cirrha (Fig. 16), appear on the subumbrellar surface at the interradii of the manubrium three days after separation. They increase rapidly until as many as thirty-five or forty appear in each interradius. An ephyra 5 mm. in diameter has eight gastric cirrha in each interradius, while the 8-mm. one has ten, and the ephyra of 15 mm. has twenty-five. These cirrha increase in number as development in the medusa continues. Development in the ephyra is completed in from six days to two weeks. Upon obtaining the bell shape, it is considered as the early "post-ephyral stage" of the medusa.

Summary and Conclusions

Investigation of the life cycle of *Dactylometra quinquecirrha,* L. Agassiz in the Chesapeake Bay has been in progress since 1935. The

results have been obtained (1) from observations made on the organism reared under controlled laboratory conditions and (2) from specimens reared under natural conditions. These observations have been supplemented by continuous investigation of the life cycle as it occurs under normal conditions.

This study has shown that the metagenetic cycle, previously reported by Agassiz and Mayer (1898), requires a period of from ten to twelve months for development from the egg to the medusa. The collection of scyphostomae and ephyrae from the waters of the Bay, as well as the successful rearing of the polyp stage under natural conditions over a period of four years substantiates the opinion of Cowles (1930) that the sea nettle breeds in the Bay. There is no evidence to support the contention that it breeds in the salter ocean water and migrates into the Bay. As was pointed out by Littleford and Truitt (1937), ephyrae have been collected in large numbers up deep creeks and inlets at times when they were not found in open Bay waters.

The common method of asexual reproduction in the scyphostoma stage is by means of pedal discs, budding being of rare occurrence. In the course of the study, it was noted that the scyphostoma had the capacity to encyst under certain environmental conditions. These cysts later produced polyps that continued development in the normal manner. Strobilization differs markedly from that known to occur in other species, in that the number of ephyral discs produced is constant at either five or six, at no time approaching the condition where a large and variable number is produced, as in *Aurellia aurita.*

The fact that the scyphostoma can live for very long periods of time was pointed out over one hundred years ago by Dalyell (1836), the discoverer of the polyp of *Aurellia.* It is of interest to note that this investigation established the fact that in *D. quinquecirrha* the scyphostoma can live for rather long periods of time. Certain individual polyps have been reared through four successive years and have undergone strobilization each summer during that time.

The ephyra, when small, lives close to the bottom and swims with the subumbrellar surface upward. After development of the manubrium and the eight primary marginal tentacles, the normal position is attained. Growth of the ephyra is a rapid process; the organism may increase ten times in size within a few days.

The life cycles of the red and white medusae are identical as regards rate of development and actual size of the morphological stages. The two "varieties" readily interbreed and the resulting cross shows no deviation from normal in its developmental history.

ACKNOWLEDGMENT

The writer wishes to express his appreciation to Professor R. V. Truitt, Director of the Chesapeake Biological Laboratory, for suggesting the problem and for his continuous advice and assistance during its prosecution; to Dr. C. L. Newcombe and Professor N. E. Phillips of the University of Maryland for helpful advice and suggestions; and to Professor W. L. Threlkeld, of the Virginia Polytechnic Institution, for his association with the investigation during the summer of 1935.

Particular thanks are due Professor E. A. Andrews, of the Johns Hopkins University, for a critical reading of the manuscript and for offering valuable suggestions.

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THE BLOCKING OF EXCYSTMENT REACTIONS OF COLPODA DUODENARIA BY ABSENCE OF OXYGEN [1]

MORDEN G. BROWN

(*From the School of Biological Sciences, Stanford University* [2])

The encysted state of a protozoön may be considered one of high stability in that little or no energy is required for its maintenance. Cells in this condition are not in any dynamic equilibrium of diverse reactions, but in a static state. This investigation is concerned with the fundamental problems of the nature of the changes from the dynamic to the static and from the static to the dynamic state as found in the encystment and excystment of protozoa.

The excystment process of the holotrichous ciliate, *Colpoda duodenaria*, is more than a reactivation of metabolic enzyme systems. The process involves a redifferentiation of protoplasmic structures, cilia, etc., along with special physical-chemical systems such as the contractile vacuole system and in addition involves the processes for escape from the cyst membranes.

Though like all ontogenetic processes, the excystment process is thus complex, it may prove amenable to analysis since the encysted organisms may be made very nearly uniform and will remain in a resting state with little or no change until reception of an excystment-inducing substance from their environment. The uniformity of the cyst preparation is obtainable since encystment as well as excystment depends on environmental conditions which may be controlled (Taylor and Strickland, 1938). A standardized, constant biological material may thus be made available for an extended series of experiments, and quantitative as well as qualitative results compared throughout the series.

The investigation into the nature of the physiological processes involved in excystment has been (1) by chemical analysis of substances which will induce the process (see Haagen-Smit and Thimann, 1938)

[1] This study comprises part of the Ph.D. dissertation (Brown, 1938*a*) and has been briefly reported at the Richmond meetings of the A. A. A. S. (Brown, 1938*b*). Equipment used throughout this research was made available directly through the courtesy of Dr. C. V. Taylor and indirectly through a grant from the Rockefeller Foundation to Stanford University for Dr. Taylor's research in chemophysical biology.

[2] Now in the Department of Zoölogy, Washington University, St. Louis.

and (2) by determination of the relations between the excystment time (the time elapsed between substitution of the excystment solution for the salt solution in which the organisms are kept and emergence from the cyst membranes) and controlled environmental factors such as concentration of the excystment solution, temperature, oxygen tension, and x-ray irradiation (see Taylor, Brown, and Strickland, 1936; Brown and Taylor, 1938; and Brown, 1938*a*). This report presents the experimental data obtained in the study of oxygen tension as a limiting factor in excystment together with a further analysis of the physical-chemical processes of excystment of *Colpoda*.

Experimental

The *Colpoda* used in these experiments were carefully cultured and selected as to interfission age, then encysted in grooves in cellophane. The cyst-cellophane preparation was then thoroughly washed and then kept in a continuously flowing, dilute, balanced salt solution. The technique of making this preparation was developed by Mr. Strickland (Taylor and Strickland, 1935) and the cysts used throughout this study were prepared by him.

The time for excystment was determined by counting the number of still encysted organisms (100 to 150 at start of each test) at intervals throughout the period of emergence from the cyst membranes. The geometric mean time was then evaluated by graphical methods as described by Brown and Taylor (1938). This geometric mean time which is equal to the median excystment time is referred to throughout this paper as excystment time.

In each experiment a series of concentrations of excystment solution, Difco yeast extract, was used. This enables one to separate the excystment processes into two periods: (1) that inversely proportional to the concentration of the excystment solution, and (2) that independent of the concentration of the excystment solution (Brown and Taylor, 1938).

The control of oxygen tension necessitated the design and construction of a special airtight excystment chamber through which gases of various composition could be passed. This chamber must be mounted on a mechanical microscope stage and fitted to a microscope of approximately 150 $\times$ magnification. The final design (Fig. 1) was the result of a long series of improvements of chambers and mechanical stages. The chamber will contain a set of six Columbia dishes which can be successively observed by rotation of the glass plate forming the floor of the chamber. The upper, stationary part is made from a large Petri dish cover which is ground into the plate. This Petri dish cover is drilled at four points, a large hole, shown in the figure through which the

microscope objective projects, and three small ones, one for the inflow of the gas mixture, one for its outflow into a ⅛-inch tube about a foot long, and one, normally sealed and close to the gas outlet tube, through which twice concentrated excystment solutions, previously brought to equilibrium with the oxygen tension being tested, could be added to equal amounts of salt solution in the excystment dishes with a negligible admixture of air. The joint between the cover and floor and all joints about the objective have been sealed with paraffin oil throughout each experiment.

In the first series of experiments, the partial pressure of oxygen was reduced to $\frac{1}{10}$ that of air (from 150 mm. Hg to that of tank nitrogen,

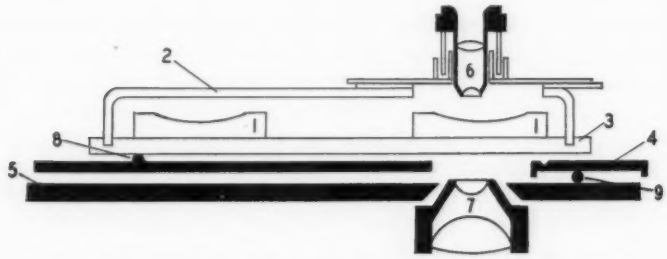

FIG. 1. Chamber and mechanical stage for studies of excystment of *Colpoda*. (1) Columbia dish containing cyst-cellophane preparation. (2) Glass cover of excystment chamber made from large, 150 mm., Petri dish. (3) Circular glass plate forming floor of chamber. (4) Moveable part of stage attached to a standard mechanical stage. (5) Microscope stage (specially constructed). (6) Microscope objective (10 × Zeiss, small size). (7) Microscope condenser. (8) One of three ball bearings in groove in moveable stage which support plate 3 and permit easy rotation of dishes. (9) One of three ball bearings between moveable stage and fixed stage.

approximately 15 mm. Hg for the tank used). The control experiments were identical in all respects with those with reduced oxygen tension except that air was flowing through the excystment chamber instead of the tank nitrogen. No differences of any kind were found when the excystment under an oxygen tension $\frac{1}{10}$ that of air was compared with the controls. A typical experimental run and two controls are shown in Figure 2. These results are in agreement with studies of respiratory rate under low oxygen tension for the free-swimming stages of the ciliates *Paramecium* (Lund, 1918, and Amberson, 1928) and *Colpoda* (Adolph, 1929); and further indicate that in none of the preceding work involving measurement of excystment time in solutions in contact with air was oxygen ever a limiting factor.

To obtain lower oxygen tensions, the nitrogen was purified by

bubbling through an acid chromous sulphate solution (M/10 Cr^{++}; pH = 2). The reduced state of the solution was maintained by the presence of amalgamated zinc prepared according to the methods of Stone and Beeson (1936). Gas exchange with the solution was facilitated by use of a sintered glass bubbler which broke up the gas stream into very small bubbles. After the air was washed out of the excystment chamber with this nitrogen and sufficient time elapsed so that equilibrium between the gaseous and liquid phase was approached, then excystment solution which had been de-oxygenated was added. There

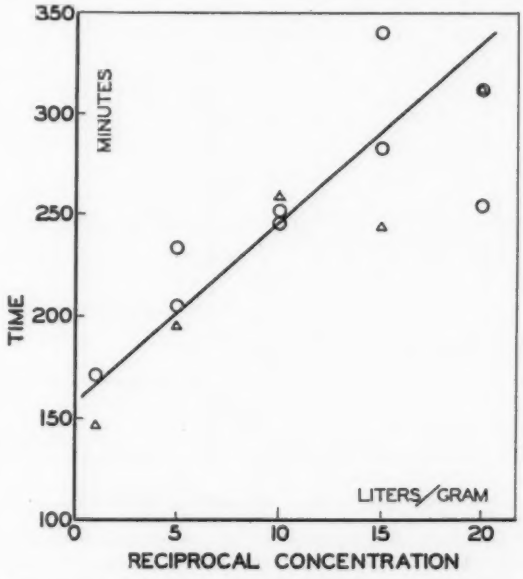

FIG. 2. Excystment of *Colpoda* at 20° C. ○, two control series; △, oxygen tension = 15 mm. Hg.

were no signs of excystment for periods of as long as 25 hours, though at 15 mm. Hg partial oxygen pressure, excystment at 20° C. would have been completed in two to three hours.

The data demonstrate much more than just a prevention of excystment by absence of oxygen, for upon admittance of air, normal excystment ensued; and further, the excystment time following the block was found to be independent of the duration of the block, independent of the concentration of the excystment solution, and equal in length to the period that was found from studies on relation between concentration

and excystment time to be independent of the concentration of the excystment solution. The experimental data for two of the series of experiments at 20° C. are shown in Figure 3. Detailed tables of these data can be found in Brown (1938*a*).

Apparently the reactions of the period in excystment depending on concentration of the excystment solution go to completion and the reactions of the subsequent periods are completely blocked in the absence of oxygen. These results are in agreement with the hypotheses previously made that the first period is controlled by a diffusion phenomenon and that the following periods at 20° C. are controlled by a reaction of

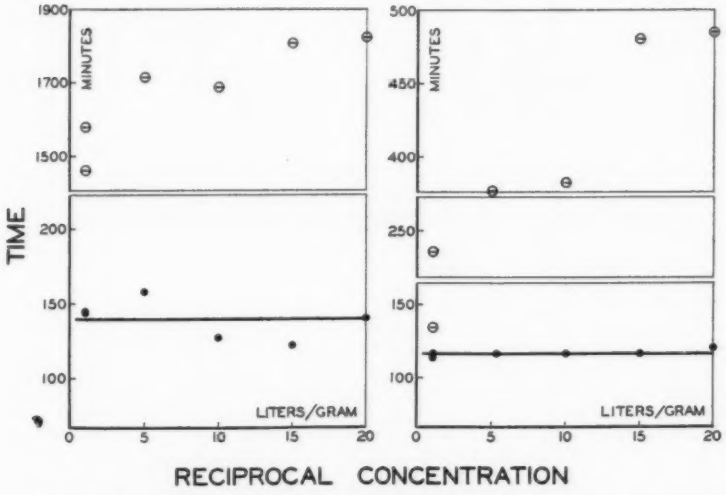

FIG. 3. Excystment of *Colpoda* at 20° C. after being blocked by very low oxygen tension. ●, time between admittance of air and emergence; ⊖, time between addition of excystment solution and emergence.

oxidative metabolism (Brown and Taylor, 1938). The fact that the reactions do not proceed in the second period in the absence of oxygen does not in itself prove that the normal limiting reaction is the oxidative metabolism—more refined experiments in which tests are made over a temperature range and in which excystment proceeds, but at a reduced rate due to oxygen tension being a limiting factor, are required.

Though the period dependent on extract concentration changes with temperature in the range 12° to 32° C. as though it were controlled by the time required for diffusion of a substance from the excystment solution, below 12° C. this period changes with temperature according

to the Arrhenius equation with a very high μ value (Brown and Taylor, 1938). From this, one might expect that a different process limits this period in the low temperature range. However, this other process, if it exists, is also independent of oxygen, for when the experiments were repeated at 11° C. it was found that the time after admittance of air for completion of excystment is at this temperature also independent of concentration and equal in duration to the period which is found by study of the relation between excystment time and concentration to be

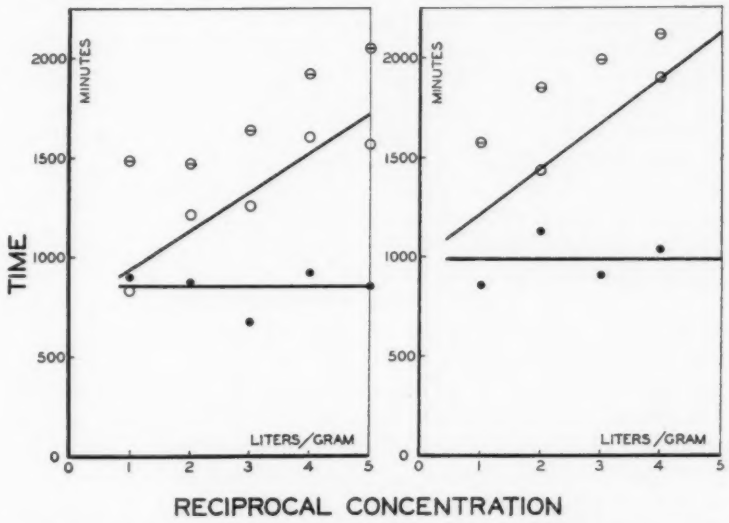

FIG. 4. Excystment of *Colpoda* at 11° C. after being blocked by very low oxygen tension. ●, time between admittance of air and emergence; ⊖, time between addition of excystment solution and emergence; ○, control-time between addition of excystment solution under aerobic conditions and emergence.

independent of concentration. Two of the low temperature series are shown in Figure 4. In the second of these series shown, the set of six dishes included four which were blocked by absence of oxygen and two to which the excystment solution was added at the time of admission of air; the conditions in the experimental and control dishes seem much more comparable in this case for it is seen that the extrapolated value for duration of the period independent of concentration coincides much more closely with the time for excystment after the block than in the cases in which the controls were run separately.

Discussion

At present four physiological periods in excystment of *Colpoda* have been sorted out by a quantitative study of excystment time under a variety of environmental conditions. Separation and characterization of the first and subsequent periods is by Brown and Taylor (1938) and this report and separation of the later periods is through the work of Taylor, Brown, and Strickland (1936) on the effects of x-ray irradiation at different stages of excystment.

The experimental characterization and physiological interpretation of these periods is briefly as follows:

I. A period whose duration is inversely proportional to the concentration of the organic constituents of the excystment solution (Brown and Taylor, 1938), and independent of oxygen tension (this report). Its duration changes with temperature as does the viscosity of the cytoplasm for a considerable temperature range (Brown and Taylor, 1938). This period is considered to be one during which diffusion of essential substances from the excystment solution takes place and possibly also an anaerobic reaction with high activation energy (Brown and Taylor, 1938, and this report).[3]

II + III + IV. A period whose duration is independent of the concentration of the excystment solution, and which is dependent upon oxygen (Brown and Taylor, 1938, and this report). The change in duration with change in temperature follows the Arrhenius equation with $\mu = 44{,}000$ calories/ mole below 15° C., 18,000 calories/mole between 15° and 25° C., and zero above 25° C. (Brown and Taylor, 1938). It is suggested that the value of $\mu = 18{,}000$ is associated with oxidative metabolism and $\mu = 44{,}000$ with an anabolic reaction of excystment (Brown and Taylor, 1938).

II. A period during which x-ray irradiation increases excystment time to the same extent as does irradiation at any time in Period I (Taylor, Brown, and Strickland, 1936).

III. A short period during which emergence from the cyst is prevented by the same x-ray dose that at other periods only delays excyst-

[3] A recent abstract of a paper by Danielli (1939) not yet published indicates that one might expect the rate of diffusion through living cell membranes to change with temperature according to the Arrhenius equation. This suggests that below 12° C. diffusion is the limiting factor in this first period in encystment rather than some postulated chemical reaction (Brown and Taylor, 1938) but that the mechanism limiting diffusion above 12° C. and below is different, i.e., a barrier of the type suggested by Danielli which requires diffusing molecules to possess greater than a certain kinetic energy in order to penetrate into the cell is limiting below 12° C., whereas cell structures that control diffusion rate according to their viscosity are limiting above 12° C.

ment or has no effect (Taylor, Brown, and Strickland, 1936). This period seems to be critical to the later building up of hydrostatic pressure which results in rupturing of the ectocyst membrane, for the irradiation prevents emergence but does not prevent the completion of differentiation of cilia or their functioning (unpublished observations of Taylor, Brown, and Strickland; see also Brown, 1938*a*).

IV. A period throughout which administration of an x-ray dose which caused a three-fold increase in excystment time if given during Periods I or II and prevented emergence if given in Period III has almost no influence on excystment time (Taylor, Brown, and Strickland, 1936). This period is considered separated from preceding ones by completion of a developmental reaction involving a substance which may be inactivated by x-ray irradiation during any preceding period.

That *Colpoda* blocked from excystment by absence of oxygen do not die or show any adverse effects for a block of at least 25 hours at 20° C. is opposite to the interpretation of some experiments with free-swimming *Colpoda* (Taylor and Strickland, 1938). In these experiments it was observed that free-swimming organisms die within a short time (97 per cent in two hours) in an unaerated dense bacterial suspension but do not die in a similar suspension which is aerated. From this it was concluded that low oxygen tensions cause death of free-swimming *Colpoda* within a few hours. This may indicate that certain enzyme systems which may be thrown out of balance by removal of oxygen and which then destroy the free-swimming organism are not activated until the second or later periods of excystment.

Summary

1. Excystment time is independent of oxygen tension down to 15 mm. Hg.

2. Excystment is blocked by very low oxygen tensions. This block is at a developmental stage between the excystment period dependent on concentration of the excystment solution and the periods independent of concentration.

3. The excystment process may be divided into four physiological periods characterized by the influence of temperature, concentration of the excystment solution, oxygen tension, and x-ray irradiation on the excystment time.

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THE RELATION BETWEEN KIND OF FOOD, GROWTH, AND STRUCTURE IN AMOEBA[1]

S. O. MAST

(From the Zoölogical Laboratory of the Johns Hopkins University and the Marine Biological Laboratory, Woods Hole, Mass.)

It is well known that amoebae usually feed on living organisms and that they ordinarily ingest several different kinds. It has, however, been demonstrated that for some species, one kind suffices for growth (Oehler, 1916, 1924; Rice, 1935; Hopkins, 1937). No observations have been made on the relation between the kind of food and the structure of amoebae. This is the main problem involved in the following experiments.

Amoeba proteus and *Amoeba dubia* grown in Hahnert solution[2] containing rice grains were fed on *Chilomonas paramecium* raised on sterile acetate-ammonium[3] and glucose-peptone[4] solutions respectively and *Colpidium striatum* raised on sterile tryptone-phosphate[5] solution. The experiments were made as follows:

Numerous amoebae were taken from vigorous cultures, passed through several separate portions of distilled water so as to remove the food, and then left in distilled water several hours. In this, many of them became stellate in form. The largest of these were selected and five of them put into each of four 6 cc. glass salt dishes containing 3 cc. Hahnert solution each. Then numerous chilomonads or colpidia which by means of the centrifuge had been passed successively through 4 separate portions of fresh Hahnert solutions were added to the solution in each, and left two hours, i.e. until the amoebae had ingested many chilomonads or colpidia, then the amoebae with as little solution as possible were transferred to clean salt dishes containing Hahnert solution. This was repeated until the solution was free of chilomonads or colpidia, after which the process of feeding and transferring was repeated and the number of amoebae in each dish recorded daily for 9 days, then 5

[1] I am much indebted to Drs. R. A. Fennell and William J. Bowen for very efficient assistance in the experimental part of this work.

[2] Hahnert solution—KCl, 4 mg.; $CaCl_2$, 4 mg.; $CaH_4(PO_4)_2$, 2 mg.; $Mg_3(PO_4)_2$, 2 mg.; $Ca_3(PO_4)_2$, 2 mg.; water, 1000 cc.

[3] Acetate-ammonium solution—$NaC_2H_3O_2$, 150 mg.; NH_4Cl, 46 mg.; $(NH_4)_2SO_4$, 10 mg.; K_2HPO_4, 20 mg.; $MgCl_2$, 1 mg.; $CaCl_2$, 1.16 mg.; water, 100 cc.

[4] Glucose-peptone solution—peptone, 8 g.; glucose, 2 g.; water, 1000 cc.

[5] Tryptone-phosphate solution—tryptone, 15 g.; KH_2PO_4, 2 g.; water, 1000 cc. 1000 cc.

of the specimens in each dish were transferred to clean dishes containing Hahnert solution and the rest discarded or used for the study of structure, after which the process of feeding, transferring, and recording

TABLE I

Growth of Amoeba fed on chilomonads and colpidia respectively. Temperature, 21°–25° C.; x, all but five discarded; * Several specimens removed for study of structure.

Food	*Amoeba proteus*											
	Number of Specimens											
	August										September	
Chilomonas in glucose-peptone solution	7	10	12	14	16	16	20	23	26	29	1	4
	5	8	8	9	6x	5	6	13	12	11	12*	
	5	9	12	15	11x	5	6	7	7	10	9*	
	5	7	6	7	6x	5	5	7	7	10	9*	
	5	6	7	4	3x	3	0					
Chilomonas in acetate-ammonium solution								5	3	0		
								5	7	0		
								5	7	1		
Colpidium	5	16	24	34	35x	5	12*	17	26	29	42*	7
	3	6	16	23	16x	5	8	13	14	20	23*	24
	4	12	24	50	90x	5	8	9	5	11	15	20
	5	8	17	27	45x	5	7	7	11	18	29	40
	Amoeba dubia											
Chilomonas in glucose-peptone solution	5	6	4	2				5	5	6		
	5	4	3	5				5	5	0		
	5	7	5	4				5	6	3		
	5	4	5	5				5	5	0		
Chilomonas in acetate-ammonium solution								5	2	0		
								5	3	0		
								5	5	2		
Colpidium	5	10	13	17	22x	5	5	5	2	0		
	5	13	24	26	44x	5	3	6	2	1		
	5	9	11	13	21x	5	6	3	3	0		
	5	12	13	12	20x	5	1	0				

was again repeated daily for 16 days. The results obtained are presented in Table I.

This table shows that both *Amoeba proteus* and *Amoeba dubia* fed exclusively either on chilomonads or colpidia increased in number, but

that the increase continued thruout the experiment only in *Amoeba proteus* fed on colpidia. It shows that some of the specimens of *Amoeba proteus* fed on chilomonads were still alive at the close of the experiment, but that the number had decreased; and microscopic examination showed that they were in very poor condition. The table shows that the specimens of *Amoeba dubia* fed on colpidia increased in number much more rapidly and lived much longer than those fed on chilomonads and it shows that for several days those fed on colpidia increased in number as rapidly as *Amoeba proteus* fed on these organisms, but that they then decreased rapidly in number and soon died. The table shows that no increase in number occurred in the specimens of either of the two species of *Amoeba* fed on chilomonads grown in acetate-ammonium solution and that they did not live so long as those fed on chilomonads grown in glucose-peptone solution.

This experiment was repeated in part several times. In some of the tests made, the colpidia used were taken from a culture which contained an unidentified mold, but no bacteria. In some of these tests, the amoebae were left with the food 2 hours, i.e. the same length of time as in the preceding experiments, but in others they were left only 15 minutes and in still others they were left 24 hours.

In the tests in which the amoebae were left with the food only 15 minutes there was no increase in number, either in those fed on chilomonads or in those fed on colpidia. The time was obviously not long enough for the amoebae to ingest sufficient food for growth. The results obtained in the tests in which the amoebae were left with the food 2 and 24 hours respectively are essentially the same as those presented in Table I. That is, in the tests in which chilomonads were used as food, the amoebae usually increased in number fairly rapidly for several days and then decreased, and in those in which colpidia were used, the increase in number continued longer and, under some conditions, doubtless would have continued indefinitely if the tests had not been closed. For example, in one test with *Amoeba proteus* fed on chilomonads, the number increased from 5 to 330 in ten days after which there was a slight increase for a few days, then a gradual decrease to zero, and in another with *Amoeba proteus* fed on colpidia there was a slow, but consistent increase in number for 34 days, i.e. thruout the entire experiment, with no indication of deterioration whatever, altho the increase during this entire time was only from 10 to 106.

The results obtained seem to demonstrate therefore that *Amoeba proteus* can grow and live indefinitely on sterile colpidia as food, but that *Amoeba dubia* cannot, and that neither can live indefinitely on chilomonads as food, but that chilomonads grown in glucose-peptone solution

are more nearly adequate as food than those grown in acetate-ammonium solution.

The chilomonads grown in acetate-ammonium solution contained much starch and little fat, while those grown in glucose-peptone solution contained considerable starch but no fat and they were much smaller than the former (Fig. 1). The difference in their food value is, therefore, doubtless due to difference in their chemical structure and content correlated with the chemical composition of the medium in which they grow. Growth in *Amoeba* is consequently not only correlated with the kind and the quantity of organism they ingest, but also with the physiological condition of the organism ingested.

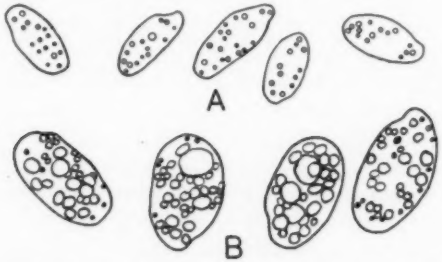

FIG. 1. Camera outlines of *Chilomonas paramecium* showing the effect of the kind of food in the culture medium on size and content. *A*, specimens taken at random from a vigorous culture in sterile glucose-peptone solution; *B*, specimens taken at random from a vigorous culture in acetate-ammonium solution; ○, starch; ●, fat. The flagella are not represented.

Note that the chilomonads grown in glucose-peptone solution were much smaller and contained much less starch and fat than those grown in acetate-ammonium solution. Growth is more rapid in the former solution than in the latter.

In the experiments on growth in *Amoeba* fed exclusively on sterile chilomonads and colpidia respectively, specimens were taken from the cultures at different times and studied in reference to behavior and structure. The results obtained are summarized in the following pages.

The specimens of *Amoeba proteus* which had fed exclusively on colpidia for several days were extraordinarily large (Fig. 2) and literally packed full of globules of fat, especially those which had fed on colpidia from the culture which contained mold.[6] They had only a few

[6] This mold contained much fatty acid but no neutral fat and the colpidia contained enormous quantities of neutral fat but no fatty acid. In fresh cultures the colpidia multiplied rapidly and became abundant in 24 hours. At this time the solution was perfectly clear and the colpidia in it contained but little or no fat. Then the solution gradually became turbid and in 4 or 5 days, mold was clearly visible and at this time the colpidia were well filled with globules of fat and each one usually contained 3 or 4 fragments of mold hyphae or spores which contained liberal quantities of fatty acid which was doubtless changed to neutral fat in the cytoplasm of the colpidia.

pseudopods and these were very short, thick, and blunt without a hyaline cap. They were only slightly attached to the substratum and moved about very slowly and irregularly, now in one direction, then in another, giving the impression of very sluggish, aimless, rolling about. Many had two nuclei. The alpha and beta granules were normal in number and structure, but the bipyramidal crystals were scarce and much

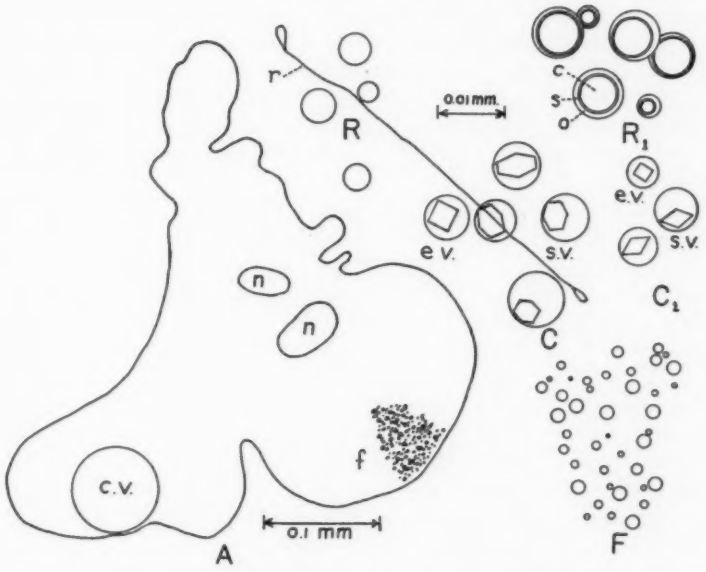

FIG. 2. Camera outlines showing the size, form, and structure of *Amoeba proteus* fed exclusively on colpidia and chilomonads respectively.

A, optical section of *Amoeba proteus* fed on colpidia; *n*, nucleus; *c.v.*, contractile vacuole; *f*, fat globules in one focal plane; *F*, same, enlarged; *C*, bipyramidal crystals in *A*; *R*, largest refractive bodies in *A* (substance in them not differentiated); *r*, one of these drawn out in the form of a fiber; C_1, bipyramidal crystals in a specimen fed on chilomonads; *s.v.*, side view; *e.v.*, end view; R_1, refractive bodies in a small area in an optical plane in a specimen fed on chilomonads (substance in these highly differentiated); *o*, outer layer; *s*, shell; *c*, central substance; *mm*, projected scale.

shorter and thicker and more truncated than usual and there were usually only a few spherical bodies and some specimens had none at all. The spherical bodies were with few exceptions very small and the substance in them undifferentiated. There was nothing in them similar to the fragile shell usually found and all the substances in them usually stained crimson with neutral red, but the central portion often appeared lighter

in color and somewhat more granular than the rest, and did not stain so readily. This substance was usually so elastic that if the bodies were released after they had been flattened by means of pressure on the cover-glass they soon assumed their original shape and it was so adhesive and viscous that if the cover-glass was pushed sidewise on the slide after the bodies had been flattened by pressure on it, the substance in them, owing to adhesions to the glass, was often drawn out in the form of a long slender fiber (Fig. 2).

It is consequently obvious that if the food of specimens of *Amoeba proteus* is restricted to colpidia great changes occur in them in reference to size, form, behavior, and structure; in fact, changes so great that if such specimens were examined without information as to their origin they would certainly be designated as a new species and probably as a new genus.

Specimens of *Amoeba proteus* which for several days had fed exclusively on chilomonads were normal in size, form, and activity; but they contained an extraordinarily large number of spherical bodies (often a thousand or more) and numerous bipyramidal crystals and very little or no fat. The spherical bodies were relatively very large and the substances in them well differentiated into a central mass surrounded with a prominent fragile shell which was covered with a thin layer of oily substance (Fig. 2). In solutions containing neutral red, the outer layer became deep red (crimson) in color, but the central portion and the shell did not stain. The spherical bodies in these amoebae were, therefore, similar to some described by Mast and Doyle (1935, p. 167) but differed radically in number, size, and structure from those found in the amoebae fed exclusively on colpidia.

The bipyramidal crystals were relatively long and but little truncated and in some specimens as many as 2 percent of them were not truncated at all (Fig. 2).

The facts that there were many more refractive bodies in the amoebae which had fed on chilomonads than in those which had fed on colpidia and that they were much larger and the substance in them much more differentiated, show that these structures are closely correlated with the kind of food ingested. They therefore support the contention of Mast and Doyle (1935, p. 291) and others that they are cytoplasmic inclusions and not cytoplasmic structures, i.e. secondary nuclei (Calkins, 1905), cysts (Taylor, 1924), Golgi bodies (Brown, 1930), mitochondria, (Horning, 1925, 1928), vacuome (Volkonsky, 1933).

Amoeba dubia usually contains relatively few crystals (some irregular or roughly bipyramidal in form with the edges and corners rounded, and some thin rectangular plate-like in form) and not much fat.

In the specimens fed on colpidia the irregular crystals decreased greatly in number and often disappeared entirely and the plate-like crystals increased considerably and there was marked accumulation of fat, altho not nearly so much as in *Amoeba proteus*. In those fed on chilomonads the irregular crystals increased greatly in number and the plate-like crystals decreased considerably and the fat usually disappeared.

There was no significant change in size, form, or activity in those fed on colpidia or those fed on chilomonads.

Summary

1. If specimens of *Amoeba proteus* are fed exclusively on colpidia, they become very large and extremely fat and sluggish and grow and multiply slowly, but indefinitely. The refractive bodies in them decrease greatly in number and size and their content becomes homogeneous and very adhesive, elastic and viscous. The crystals decrease in number and become shorter and more truncated.

2. If they are fed exclusively on chilomonads, they grow and multiply for several days, then decrease in number and soon die, but they live longer if the chilomonads have grown in glucose-peptone solution than if they have grown in acetate-ammonium solution. The refractive bodies increase greatly in size and number and the content of these bodies becomes sharply differentiated; the bipyramidal crystals increase in number and become less truncated, and the fat decreases in quantity.

3. If specimens of *Amoeba dubia* feed exclusively on chilomonads, they multiply for a few days, then cease and soon die. The irregular-shaped crystals increase and the plate-like crystals decrease considerably in number and the fat disappears.

4. If they feed exclusively on colpidia, they multiply more and live longer than if they feed exclusively on chilomonads, but they do not live indefinitely. The plate-like crystals increase in number and the irregularly shaped crystals usually disappear entirely and the fat increases in quantity, but not so much as it does in *Amoeba proteus*.

5. *Amoeba* is in reference to form, size, behavior, and structure closely correlated with the kind of organisms it eats and their physiological condition.

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THE EFFECT OF ELECTRIC CURRENT ON THE RELATIVE VISCOSITY OF SEA-URCHIN EGG PROTOPLASM [1]

C. A. ANGERER [2]

(From the Zoölogical Laboratory, University of Pennsylvania,[3] and the Marine Biological Laboratory, Woods Hole, Mass.)

Becquerel (1837) was the first investigator to study the action of electricity on protoplasm of single cells. [The greater part of the literature treating of the effect of electric current on cyclosis may be obtained from Ewart (1903).] The conclusion to be drawn from the literature is that electric current, depending on the current density employed, produces a progressive decrease in, followed ultimately by cessation of, cyclosis providing the current flows for a sufficient interval of time. However, Velten (1876), Ewart (1903) and Koketsu (1923) observed an initial increase prior to the characteristic progressive slowing of cyclosis. The results obtained by Brücke (1862) on human leucocytes, Chifflot and Gautier (1905) on *Cosmarium*, Bayliss (1920) on *Tradescantia* and *Amoeba* by the Brownian movement method and Bersa and Weber (1922) on *Phaseolus* by the centrifuge method are in essential agreement, i.e., electric current produces an increase in protoplasmic viscosity.

This investigation was undertaken to continue and extend the study of electric current as a stimulating agent to some type of protoplasm other than that of the protozoan cells *Amoeba dubia* and *A. proteus* already studied (1937).[4] It is of interest to know whether the protoplasm of such distantly related biological groups, e.g., *Amoeba* and the unfertilized eggs of *Arbacia*, responds to this stimulating agent in a comparable manner.

Material and Methods

The experiments were performed on the unfertilized eggs of the sea-urchin, *Arbacia punctulata*. The eggs were treated according to method "3" as described by Just (1928). Eggs were shed in about

[1] This investigation was aided by a grant-in-aid from the Society of the Sigma Xi.

[2] Now of the Dept. of Physiology, The Ohio State University, Columbus.

[3] I wish to express my profound gratitude to Professor C. E. McClung for his many kindnesses during the present investigations.

[4] Our own studies on amoebae are now nearing completion and a detailed report will appear shortly.

250 cc. of sea water and washed once in an approximately equal volume of the medium.

The eggs were subjected to either direct or alternating electric current, according to the experiment in question, in a celluloid trough, the sides of which were perfectly milled and thus parallel to the lines of current flow. The current was applied through a $Zn/ZnSO_4/$ sea-water-in-agar system, which, in turn, was in circuit with a reversing switch, rheostat and milliammeter. The agar bridges were cut to fill the ends of the trough completely and were finally sealed in place by means of hot agar. The available electrode surface, i.e., the cross-sectional area of the available medium in the trough, was 40 mm^2. The source of the electric current was the regular service line (110 volts) running into the laboratory.

The protoplasmic viscosity was determined by the centrifuge method. The handle of an Emerson hand centrifuge, when turned at the rate of one revolution per two seconds, developed a centrifugal force of 2,531 times gravity, after allowance was made for the depth to which the eggs settled in the centrifuge tube when cushioned on an isosmotic (0.73 m.) sucrose solution. The eggs were centrifuged until 80 per cent, or more, showed a fine hyaline band 2/15 the diameter of the egg appearing between the oil cap and the yolk granules. (This fraction was equal to one division of the arbitrary scale of the ocular micrometer employed in these experiments.) The time in seconds necessary to move the yolk granules the specified distance, and thus show the requisite width of the hyaline band at the centripetal pole of the egg, is designated the "centrifuging value." This is the end-point to which all experimental centrifugalizations are referred.

The experiments were conducted in the following manner. Each batch, i.e., eggs from one female, was tested for 'normalcy.' A batch of eggs was declared 'normal' if, on sampling, 95 per cent or more showed membrane elevation after insemination and 80 per cent or more, showed the desired width of the hyaline area when centrifuged for 60 seconds at room temperature (19°–24° C.). These conditions prevailing, approximately uniform quantities of eggs were placed in the stimulating trough and while the eggs were more or less suspended, an electric current of known intensity and duration of flow was admitted. At the cessation of the current the eggs were immediately pipetted into the centrifuge tube, which contained a known depth of isosmotic sucrose solution, and were centrifuged respectively for various known periods of time. The lowest value to which the interval of time elapsing between the cessation of the application of the stimulating agent and incipient centrifugalization could be reduced was 7 seconds. There was no

apparent difference between this and the 10-second interval which was used throughout these experiments unless otherwise stated.

The points of curves *A* and *B* (Fig. 1) which represent centrifuging values plotted as functions of the time of exposure to the electric current in question were obtained in the following manner. After preliminary tests in which various constant current densities were studied, when applied for varying intervals of time, it was apparent that a current density [5] of 0.005 amperes/mm.^{2} served best to illustrate the results and this density was employed throughout these experiments.

On the basis of the above procedure the curves were developed as

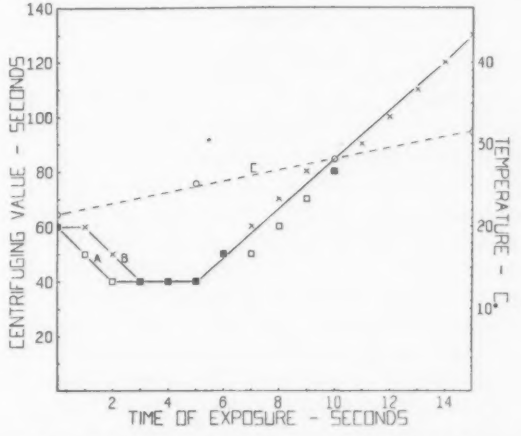

FIG. 1. Centrifuging time in seconds (viscosity) of *Arbacia* egg protoplasm vs. time of exposure in seconds to alternating (curve *A*) and direct (curve *B*) electric current. Curve *C* represents the rate of thermal increase to which the stimulating trough is subjected vs. time of exposure to either type of current. Current density = 0.005 amperes/mm.^{2}; pH 8.2; room temperature = 21.5° C.

follows. In the early experiments several batches of eggs were necessary in order to work out a complete curve. With more experience one batch of eggs sufficed for the determination of the points for any one curve. Twelve curves for direct (*B*) and ten curves for alternating (*A*) current were thus worked out. Finally, with these data as a basis, all points for both alternating and direct current curves were obtained by employing one batch of eggs. This procedure was repeated for three different batches of eggs and thus assured relatively constant conditions of material, temperature (21.5° C.), etc. Hence fewer experiments were performed in the latter instance but these data were substantiated by the

[5] In a preliminary note (1938) amperes/cm.^{2} should have read amperes/mm.^{2}.

more detailed experiments performed during the early part of the work. Under the conditions of the experiments no point on the curves deviated more than ten arbitrary units in centrifuging value in repetitive tests.

Eggs were not used for experimental purposes after being shed longer than three hours. The viscosity of the main protoplasmic mass, as determined by the method here employed, does not undergo any appreciable change during this three-hour period (Goldforb, 1935; Angerer, 1937).

There was no observable difference when experimentally treated eggs were compared with their controls for membrane elevation and cleavage.

Results

Direct Current

When sea-urchin eggs are subjected to a current density of 0.005 amperes/mm.^{2} flowing for varying known intervals of time, there is, in the majority of experiments, no observable change in the centrifuging value (curve B) after one second of continuous exposure.[6] On continuous application of the current for two seconds, there is, in all experiments, a decrease in the centrifuging value; while with three seconds of continuous exposure the viscosity decreases from the control centrifuging value of 60 to a minimum value of 40 arbitrary units, i.e., a decrement of 33 per cent in three seconds. There is no further change in viscosity for the next four or five seconds, respectively, of continuous exposure to the current.[7] However, if the current is allowed to flow for six seconds, the centrifuging value increases from a transient minimum value until after seven seconds of constant exposure the centrifuging value is identical with that of the control. There is a progressive increase on further exposure, so that after fifteen seconds, when these experiments were discontinued, the centrifuging value had increased 225 per cent above the previous minimum value.

Alternating Current

When the centrifuging values are plotted as functions of the time of exposure in seconds (curve A) to alternating current of 0.005 amperes/

[6] In a few experiments the viscosity at the end of this period of time was found to show a slight decrease which was never greater than a centrifuging value of ten seconds.

[7] Occasionally eggs, after exposure to electric current, showed a tendency for the intracellular granules to stick in the cortical area. This condition, though infrequent, was confined, more particularly, to eggs from certain females. Though these eggs were discarded in the final count, since the behavior of the main mass of the protoplasm was of chief interest, their number was not of such magnitude as to affect appreciably the results.

mm.[2] there is, in all experiments, after one second of constant current flow a decrease in the viscosity of the protoplasm as measured by the centrifuging value. This value is decreased further when the eggs are exposed to the current for two seconds, while after three seconds the centrifuging value undergoes no further decrease but levels at the new minimum value which is 67 per cent of the control. This minimum value is maintained after continuous exposure for three, four and five seconds, respectively.[7] Continuing the exposure to the stimulating agent further, there is, after five seconds, a perceptible increase in the viscosity value. Thereafter, with each successive second of exposure to the electric current a progressive increase is noted in the centrifuging value. The increment is of the same value as that recorded for direct current (curve *B*), namely 225 per cent in ten seconds. The results recorded (curve *A*) were discontinued in this experiment after ten seconds exposure to alternating current because of the expiration of the time limit set upon the use of shed eggs.

It was of interest, in view of the high resistance at the sea water-agar interface, to determine the thermal change occurring within the stimulating trough as a result of the passage of an electric current of specified density. The main coördinates of the broken-line curve *C* (labeled at the right and lower sides of Fig. 1) represent temperature as a function of time during which the eggs contained in the trough are exposed to the thermal effect induced by passage of the electric current. The temperature data were obtained by a specially constructed, direct reading thermometer, the bulb of which was of such size as to be submerged completely when immersed in the stimulating trough.

That thermal effects of the magnitude present during the course of these experiments have no observable effect on the centrifuging value is shown on immersing eggs in sea water which has been warmed previously to 32° C. When eggs are exposed to this temperature for greater intervals of time (e.g., 20 seconds) than that to which they are subjected during the course of these experiments and are simultaneously centrifuged with eggs serving as controls, it is found that the centrifuging values are identical. Heilbrunn (1924), though not primarily interested in this phase of the question, states in the protocol (p. 192) of his experiments on heat coagulation in sea-urchin eggs that after five minutes exposure to 32.9° C. the heat-treated eggs were found to show the same width of the hyaline area as the controls.

Experiments were conducted to test whether varying the quantity of eggs suspended in unit volume of sea water in the specified electric field had any tendency to alter the shape of the electric current-viscosity curves. Batches of eggs were allowed to settle under the influence of

gravity in a four-inch finger bowl and minimum amounts of sea water plus the relatively concentrated eggs were picked up by means of a regular medicine dropper. Various points on the electric current-viscosity curves were investigated using one, ten and twenty drops respectively of the egg suspension in unit volume of bathing medium. In the various concentrations of eggs employed there is no observable difference in the centrifuging values other than that which is within the range of experimental error.

Discussion

When either direct or alternating electric current of the intensity employed in these experiments is used as a stimulating agent, there is initially a transient decrease followed by a progressive increase in the viscosity of the protoplasm of sea-urchin eggs. An ultimate increase in the centrifuging value is in line with the literature (see introduction); though no lucid evidence is to be found in favor of a transitory decrease in viscosity prior to the ultimate increase.

In view of Heilbronn's (1914) results on the attempt to correlate cyclosis in terms of viscosity data, it is justifiable to consider only, at the present time, data as obtained from the methods of Brownian movement and centrifugalization. Brücke (1862), Kühne (1864), Chifflot and Gautier (1905), and Bayliss (1920) have observed a decrease or stoppage of Brownian movement on passage of an electric current through the cell. These data, insofar as a definite statement as to the experimental procedure is given, were obtained during the actual passage of electric current through the material in question and not immediately thereafter as in the experiments here reported. There may be some criticism of studying Brownian movement during the actual passage of the current since cytoplasmic granules undergoing electrophoresis lose their characteristic trembling movements (unpublished results). Mast (1931) should be consulted in this connection. Bersa and Weber (1922), using the centrifuge method, observed an increase in the viscosity of the protoplasm of *Phaseolus* on the passage of electric current for relatively long periods of time. It would be of interest to ascertain data for shorter intervals of time.

There is no difference in the results whether one employs alternating or direct current providing identical current densities are employed (compare curves *A* and *B*). Alternating current tends to be more effective initially owing, apparently, to the greater shearing effect produced by the protoplasmic granules suspended in an oscillating electric field which would tend to break down the protoplasmic structure. This effect may be reenforced by the apparently thixotropic character of

protoplasm. For a review of the literature on thixotropy in living cells see Angerer (1936).

The question arises as to the congruity of applying a stimulating agent for the duration of a few seconds while a minimum of 50 seconds is required for obtaining the viscosity determination. When varying intervals of time are permitted to elapse from the cessation of the electric current to incipient centrifugalization, the results obtained are found not to vary for at least two minutes.

The data presented here are in accord with the known facts concerning the action of certain stimulating agents on sea-urchin egg protoplasm. Heilbrunn and Young (1930) and Angerer (1937),[8] employing respectively ultra-violet radiations and mechanical agitation, found a transitory liquefaction prior to an ultimate increase in viscosity. Similar results were obtained for ultra-violet radiations (Heilbrunn and Daugherty, 1933), mechanical agitation, electric current and suddenly applied thermal increments (Angerer, 1936, 1938, 1940) on *Amoeba* protoplasm. To explain their results, Heilbrunn and Daugherty (1933) proposed a theory in terms of colloid chemical changes in protoplasm; for a detailed review of this theory one is referred to Chapter 37 of Heilbrunn's book (1937).

Summary

1. The centrifuge method was used to determine the viscosity of sea-urchin egg protoplasm after exposure to either direct or alternating electric current to a current density of 0.005 amperes/mm.^{2} for various known intervals of time.

2. There is, on exposing eggs to either direct (curve *B*) or alternating (curve *A*) current, a transient decrease followed ultimately by a progressive increase in the centrifuging value (Fig. 1).

3. Since the data for the action of electric current, as employed in these experiments, show a striking similarity to those results as obtained by the use of certain other stimulating agents on *Amoeba* and *Arbacia* egg protoplasm, it is suggested that the mechanism offered by Heilbrunn (1937) may be applicable here.

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[8] Footnote p. 340.

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DEVELOPMENT OF EYE COLORS IN DROSOPHILA: PRODUCTION OF v^+ HORMONE BY FAT BODIES [1]

G. W. BEADLE, E. L. TATUM AND C. W. CLANCY

(From the School of Biological Sciences, Stanford University, California)

Of the two diffusible substances known to be concerned in the production of eye pigments in *Drosophila*, only v^+ hormone is produced by fat bodies (Beadle, 1937). This was demonstrated by transplantation experiments. Attempts to extract this hormone from larval fat bodies were unsuccessful and it was therefore concluded that the hormone is produced after the time of puparium formation. It is the purpose of this paper to summarize additional experiments designed to determine when and under what conditions fat bodies produce this hormone. Unless otherwise indicated, fat bodies were taken from wild-type larvae or prepupae. All tests for v^+ hormone were made by using vermilion brown flies as described by Tatum and Beadle (1938). The few tests made for cn^+ hormone were made in a similar way using cinnabar brown flies.

Larval Fat Bodies

Although v^+ hormone could not be extracted from larval fat bodies with Ringer's solution at 100° C., it was felt that the hormone as such might nevertheless be present but in such a state that it was not extracted by the method used. Accordingly, several additional methods of extraction have been used.

Fifty sets of dissected fat bodies were heated in distilled water, oven-dried, and extracted with chloroform. The chloroform-insoluble material was taken up in hot Ringer's solution and injected into vermilion brown test larvae. The results were negative (8 flies). Since the hormone is known to be chloroform-insoluble and water-soluble, these tests confirm those previously made. Other tests in which the dissected fat bodies were ground with powdered silica were likewise negative (3 flies). Alternate freezing of fat bodies (in an acetone and solid CO_2 mixture) and thawing for six successive times failed to yield any hormone in subsequent extracts made with hot Ringer's solution (6 flies).

Digestion of larval fat bodies with trypsin failed to liberate any

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hormone. In one experiment 20 sets of fat bodies from mature larvae were incubated for 24 hours at 37° C. in 0.03 cc. of a solution of 0.5 per cent of $NaHCO_2$ and 0.025 per cent of trypsin made up in Ringer's. The clear solution obtained after heating and centrifuging through a microfilter gave negative results (20 flies). Appropriate controls showed that under these conditions the trypsin used was active in digesting casein and that it did not alter the activity of concentrated extracts of the hormone. Observations showed that the trypsin-treated fat bodies were visibly broken down.

Further attempts to determine whether any v^+ hormone is present in larval fat bodies were made by freezing such tissues with solid CO_2 and then transplanting them to test animals. This was done by taking up the fat body tissue in a regular transplantation pipette (Ephrussi and Beadle, 1936) and then placing the shaft of the pipette in contact with a small piece of solid CO_2. The temperature actually attained by the tissue itself was not determined; it was without question well above that of the CO_2. Ten flies to each of which a section of fat body (attached along one margin to the salivary gland) had been transplanted after being frozen three times, showed little or no eye-color modification. Other experiments using a more or less similar technique were made with fat bodies immersed in boiling water before transplantation. Considerable difficulty was encountered in doing this, but by coating the inside of the pipettes with a thin film of agar, drawing up the fat-body tissue, and then immersing the pipette in boiling water, a number of successful transplants were made. Of seven test animals to which such heated fat bodies were transplanted, six were quite negative. The seventh showed a color modification of 2.5 (medium response—see Tatum and Beadle, 1938, for significance of color values). This exceptional animal was undoubtedly one to which by mistake an unheated fat body had been transplanted. Because of the technical difficulty of making such transplants such an error could easily have been made. Living fat bodies were transplanted as controls for both the frozen and heated series. Eight such control transplants gave a mean color value of 2.4 (1.8 to 3.0). A single control transplant gave a negative test, presumably due to failure of the operation.

These experiments agree with those previously made and indicate that little or no v^+ hormone is present in larval fat bodies prior to puparium formation, and consequently that the major portion of the hormone produced by such tissues is elaborated after puparium formation. It is possible that a small amount of hormone is produced before this time but in too small an amount to be detected by the methods used.

Fat Bodies of Prepupae

Preliminary experiments indicated that v^+ hormone is present in prepupal fat bodies and can be extracted from them during this stage. Several series of extractions of prepupal fat bodies taken from animals of various ages were therefore made. In each case 20 sets of fat bodies were heated in 0.03 cc. of Ringer's solution and the solution removed by centrifuging through a microfilter. One series, using prepupae from the Oregon-r wild-type stock gave the results shown in Table I.

Table I

Age in Hours after Puparium Formation	Number of Test Animals	Eye Color, Mean and Range
0–1	11	0.1 (0.0–0.4)
3–4.5	14	0.0
6–8	10	2.2 (1.5–3.0)
7–9	15	2.3 (0.0–3.2)
10–11.8	10	0.8 (0.0–2.7)

There is no apparent reason why the 3–4.5 hour prepupal fat bodies gave negative results. A number of other tests indicate that the results are generally erratic for young prepupae. Thus a separate set gave a mean color value of 1.3 for an extract of 0–1 hour prepupal fat bodies. A series of tests of prepupae from the Canton-S wild-type stock gave the results shown in Table II.

Table II

Age in hours	Number	Eye Color
larval	10	0.0
0–1	10	0.3 (0.1–0.6)
1.8–3.5	8	0.7 (0.0–1.6)
8–9.5	8	1.4 (0.7–2.0)

An additional experiment using 10–12-hour Oregon-r prepupae gave a test of 2.3 (6 animals 1.3–3.0). It should be pointed out that the tests of older prepupae are unreliable because of the impossibility of being sure of getting all of the fat body tissue. At this time the fat bodies are undergoing the breakdown process characteristic of metamorphosis.

These results suffice to show that the hormone is present in fat body tissue and may be extracted over most of the prepupal period. Because of the several difficulties involved in such tests as these, the results are only roughly indicative of quantitative relations.

Correlation of Hormone Production and Puparium Formation

Various attempts have been made to alter the conditions so that larval fat bodies would produce v^+ hormone. Unheated wild-type larval fat bodies were allowed to stand in Ringer's solution for 5 to 6 hours at room temperature. Extracts of these gave negative results. Several series of 48-hour-old wild-type larvae were subjected to semi-starvation conditions by transferring them to 0.25 per cent dry brewers' yeast in 1 per cent agar as described by Beadle, Tatum and Clancy (1938). This reduced food supply prolongs larval life. Extracts of the fat bodies of such delayed larvae made just prior to puparium formation failed to show the presence of hormone. On the assumption that enzymes might be involved in the production of v^+ hormone by the fat bodies, pupal fluid from vermilion brown animals selected from 0 to 30 hours after puparium formation was injected into wild-type larvae 117–124 hours after egg-laying. Three to 7 hours after these injections were made the fat bodies of the hosts were removed and extracted with hot Ringer's. These extracts were negative in tests for v^+ hormone. A similar experiment in which vermilion brown pupal fluid was injected into 92–97-hour wild-type larvae gave negative results in tests of fat body extracts made 23–25 hours later.

A marked delay in puparium formation brought about by subjecting mature larvae to low temperature apparently does not break down the synchronism between hormone production and puparium formation. An experiment in which wild-type mature larvae were kept at 8–10° C. for 18.5 hours showed that a Ringer extract of fat bodies of 0–1-hour-old prepupae taken at the end of this time gave a mean color value of 0.3 when tested in 11 vermilion brown animals. A comparable extract made from prepupal fat bodies from mature larvae kept continuously at 25° C. gave an average color value of 0.6 (11 flies). Considering the low values obtained from these two extracts and the variation (0.1–0.7 and 0.1 to 0.8 respectively), this difference cannot be regarded as significant.

Prepupal fat bodies 0–1 hours after puparium formation apparently do not continue hormone production when explanted to Ringer's solution. In one experiment 20 sets of such fat bodies were placed in 0.03 cc. of Ringer's solution and allowed to stand at 22° C. for 27–28 hours. At the end of this time a hot-Ringer extract gave a color value of 0.4 (range 0.0–0.7, 14 animals). A control series extracted in a similar way immediately on dissection gave a color value of 0.6 (range 0.1–0.8, 11 animals). The explanted fat bodies did not undergo the breakdown processes characteristic of metamorphosis.

Superfemales of *Drosophila* (individuals with 3 X chromosomes and 2 sets of autosomes) are known to show a delay of one to three days in puparium formation as compared with their normal sisters (Brehme, 1937). During this period, subsequent to puparium formation by their sibs, there is relatively little growth of the superfemale larvae. Extracts of fat bodies of such superfemale larvae taken shortly before puparium formation show that v^+ hormone is present at this time. Thus an extract of 20 sets of fat bodies from mature superfemale larvae in 0.03 cc. of Ringer's solution gave a mean eye-color modification of 0.7 (range 0.0–1.0, 10 animals). Extracts of prepupal fat bodies of superfemales are likewise positive. It is clear, then, that under the particular set of developmental conditions of superfemale larvae the synchronization of fat-body hormone production with puparium formation characteristic of normal larvae is broken down. This shows that the two processes are not inseparably associated at least as regards their time sequence. The mechanism by which the two processes are normally related, however, is entirely a matter of conjecture at the present time.

While under none of the environmental and experimental conditions to which normal larvae were subjected was there any appreciable production of v^+ hormone by the fat-body cells prior to puparium formation, the fact that the sequence of these two processes is modified by the genic imbalance characteristic of superfemales suggests that it might be possible to induce the formation of hormone by cells of this tissue before puparium formation in normal larvae if the proper conditions were brought about. Certainly this possibility is not excluded by any of the work reported in this paper.

In order to determine whether or not fat bodies might have any effect on the eye-color hormones in vitro, an experiment was made in which fat bodies were explanted to a Ringer's solution containing partially purified v^+ and cn^+ hormones. As a control, fat bodies heated for several seconds at 100° C. were allowed to stand in a similar solution of the hormones. In both cases the fat bodies were kept in the solution for 4 hours at room temperature. The results are shown in Table III.

Living fat bodies appear to have no significant effect on the hormones in solution. Since the hormones may be inactivated through oxidation in the presence of certain enzymes present in the organism (Thimann and Beadle, 1937), it may be concluded that the fat body either does not contain or does not liberate such enzymes under the conditions of this experiment.

Relation of the Fat Body to the Starvation Effect

It has been shown that low food level at a certain period of development modifies vermilion flies in some manner such that they produce v^+ hormone (Khouvine, Ephrussi and Chevais, 1938; Beadle, Tatum and Clancy, 1938). Normally such flies produce little or no v^+ eye-color hormone. Since this modification evidently must be due to some alteration in metabolism, attempts have been made to determine what tissues or organs might be involved. It has been found that the fat body is modified by subjection of larvae to low food.

Larvae were transferred from full food to low food at about 48 hours after egg-laying and allowed to complete larval development under these conditions. The methods of inducing an eye color modification in this way are described in the papers referred to above. Fat bodies taken from mature larvae which had been subjected to such semi-starvation conditions were transplanted to vermilion brown larval hosts

Table III

	Test for v^+ hormone		Test for cn^+ hormone	
	Unheated	Heated	Unheated	Heated
Number of tests	11	11	8	9
Mean eye color	3.0	3.1	2.5	2.4
Range	2–3.5	3.0–3.5	2.5	2.0–2.5

grown under standard full-food conditions. In one experiment in which fat bodies from vermilion brown larvae grown on low food were transplanted, 21 host animals eclosed. Of these, 16 showed an eye-color modification (mean 0.8, range 0.1–2.0). The remaining 5 were negative, possibly because of unsuccessful operations. Since the fat body normally breaks down during metamorphosis there is no easy way of checking for the presence of implanted tissue. In another series fat bodies from vermilion larvae subjected to a low food level were transplanted to vermilion brown test larvae. Ten animals developed and all showed a positive effect of the implant (mean eye color 1.3, range 0.8–1.9).

Since it is well established that fat bodies of fully fed vermilion (or vermilion brown) larvae give negative results when transplanted to vermilion brown hosts, it is evident that low food of the kind used so modifies the fat body that it subsequently produces v^+ hormone. These results have been checked by direct extraction of the hormone from prepupal fat bodies. Extraction of fat bodies of mature vermilion brown

larvae that had been subjected to low food conditions yielded solutions that were negative in tests for v^+ hormone. Fat bodies from prepupae (4.5–6.5 hours after puparium formation) were extracted with hot Ringer's solution. This extract gave a slight but definitely positive modification of the eyes of vermilion brown test animals (11 flies, eye color 0.1–0.2). It appears that in such larvae, as in normal wild-type larvae, the fat body produces v^+ hormone subsequent to the time of puparium formation.

Preliminary studies have indicated that subjection of larvae to low food conditions brings about changes in the cytoplasmic inclusions of the fat body cells. These changes may possibly be correlated with the production of hormone by vermilion larvae which have been grown under semi-starvation conditions. Since these investigations are as yet incomplete, discussion of them will be deferred.

Malpighian tubes of wild-type larvae are known to contain v^+ hormone and there is evidence that they produce this substance. In order to determine whether the low food level might also have an effect on these organs, Malpighian tubes from semi-starved vermilion brown (or vermilion) larvae were transplanted to normal vermilion brown test larvae. It was discovered that tubes from larvae subjected to a low food level tend to kill the hosts to which they are transplanted. Presumably the tubes accumulate toxic substances under such conditions. In a preliminary series four mature recipients showed no eye color modification. In this series, however, no dissections were made to determine whether the implant was present. A second series in which sets of four Malpighian tubes from mature semi-starved vermilion were transplanted to vermilion brown test larvae, nine adult recipients were obtained which dissections showed to contain implanted tubes. Eight of these showed a relatively weak eye color response (0.5) indicating that hormone was present or was produced—the ninth was negative.

It appears that the Malpighian tubes of vermilion larvae contain or produce some v^+ hormone under the semi-starvation conditions to which these larvae were subjected. The effect, however, seems to be less strong than that on the fat bodies. It is possible that the hormone released from larval Malpighian tube transplants represents accumulation and is not produced by the tubes themselves. It does not seem probable that the hormone is produced by the fat body, although we have not entirely excluded the possibility that the larval fat body produces hormone at a low rate. The fact that no hormone (or very little) accumulates in the larval fat body argues that if it is produced in this tissue during larval life, it must diffuse out approximately as fast as it is formed.

SUMMARY

Under normal genetic and environmental conditions fat-body cells produce v^+ hormone after the time of puparium formation but not before. Attempts to induce hormone production by fat-body tissue before puparium formation were unsuccessful. Since it is shown that larval fat bodies of mature superfemale larvae contain v^+ hormone, however, it is clear that the normal sequence of puparium formation and hormone production is not a necessary and invariable one.

Active solutions of v^+ hormone are readily obtained by extracting prepupal fat bodies over practically the entire period of prepupal development.

It is shown that the so-called "starvation effect" on eye pigmentation involves a modification of genetically vermilion fat body cells such that they produce v^+ hormone, whereas normally they are unable to do so. It is possible but not definitely established that a somewhat similar modification is brought about in cells of the Malpighian tubes by semi-starvation of larvae.

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EFFECT OF DIET ON EYE-COLOR DEVELOPMENT IN DROSOPHILA MELANOGASTER [1]

E. L. TATUM AND G. W. BEADLE

(From the School of Biological Sciences, Stanford University)

Vermilion brown (*v bw*) larvae of *D. melanogaster* placed on a low food level diet produce v^+ eye-color hormone, and therefore, as flies, develop pigmented eyes (Khouvine, Ephrussi and Chevais, 1938). Beadle, Tatum and Clancy (1938) showed that larvae are affected in this way by low food level during a certain sensitive period lying between 60 and 70 hours from egg laying. Khouvine, Ephrussi and Chevais reported that sugar added to the starvation diet inhibits the starvation effect. Their work, however, did not eliminate the possibility that the sugar effect was associated only indirectly with hormone production in the flies, possibly through the intermediation of growing yeast or other micro-organisms. We have investigated the effects under aseptic conditions of various supplements to a low yeast diet on the growth and eye-color development of vermilion brown animals. Under these conditions carbohydrates and related substances inhibit the starvation effect, while proteins and amino acids do not. The present paper summarizes these results.

Experimental

Culture and Methods

The aseptic cultures of vermilion brown larvae used throughout this work were obtained by a slight modification of Baumberger's (1919) alcohol sterilization method. Eggs were collected over a 2- to 3-hour period on freshly autoclaved standard corn-meal molasses agar, without added yeast. Shortly after collection 20 to 30 eggs were picked up on a single small sterilized glass rod flattened at the end. The rods with the eggs were then placed individually in small sterile vials containing 85 per cent alcohol. After 10 minutes the rod was removed and the eggs were pushed off onto the sterile test medium, using ordinary bacteriological methods to insure sterility. All cultures were incubated at 25° C. unless otherwise stated.

The standard starvation food contained 1.5 per cent agar and 0.5 per cent Fleischmann's dry brewers' yeast made up with distilled water.

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Ten cc. of this mixture and the desired amounts of the various supplements were placed in 35 cc. vials which were stoppered with cloth-covered cotton plugs. After sterilization in the autoclave the vials were cooled and agitated, and finally slanted so that the solid yeast remained suspended throughout the medium. Routine checks of sterility were made after pupation of the larvae by streaking a loopful of the medium onto yeast extract-glucose agar. Any vials which were not bacteriologically sterile at this time were discarded.

Cultures were observed every 24 hours, so that the time to emergence of the flies was accurate only within this period. The observed prolongation of larval and pupal life as compared with the normal 215 hours on full food is given in days from egg-laying. The delay actually represents prolongation of larval life, since Beadle et al. (1938) showed

TABLE I

Influence of yeast concentration and temperature on the starvation effect. Basic medium: 1.5 per cent agar.

Temperature °C.	Yeast concentration (per cent)											
	0.5			1.0			3.0			5.0		
	Days to emergence	No. of adult flies	Eye color	Days to emergence	No. of adult flies	Eye color	Days to emergence	No. of adult flies	Eye color	Days to emergence	No. of adult flies	Eye color
17°	22–25*	7	3.5–4.5	27–29	22	3.5–5.0	24–26	39	1.0–3.0	24–26	35	0.5–2.5
25°	11–13	33	2.5–3.5	10–11	15	0.5–1.5	10	20	0.0–0.2	9–10	42	0.0
28°	10–11	26	0.5–1.2	9–10	19	0.0	8–9	29	0.0	8–9	34	0.0

* Normal developmental time on full food is 9 days (215 hours) from egg-laying.

that duration of pupal life is practically constant under all conditions. After emergence of the flies the intensity of pigmentation of the eyes was graded according to the scale of eye-color values described by Tatum and Beadle (1938). These values have a definite relation to the amount of hormone available to the fly, but for simplification all results are given only as color values. It should be remembered that the increased intensity of eye pigmentation resulting from starvation involves the actual production of v^+ hormone (Beadle et al., 1938).

Effect of Yeast Concentration and Temperature

In order to determine the most suitable conditions for the starvation effect, series with varying yeast concentrations were incubated at different temperatures, 18°, 25° and 28° C. The results are given in Table I. It was found that 0.5 per cent dry yeast at 25° C. was most

TABLE II

Influence of carbohydrates on the starvation effect. Basic medium: 0.5 per cent brewers' yeast in 1.5 per cent agar.

	Carbohydrates added (2 per cent concentration)			
	None	Starch	Sucrose	Glucose
Delay in days	2–3	2–5	1–4	1–4
Number of flies	9	62	67	45
Eye color	2.0–3.5	0.0–0.5	0.0–0.3	0.1–1.0

suitable, both for the intensity of the effect and for the developmental time required. Lower concentrations of yeast at this temperature gave somewhat stronger effects, but mortality was higher. The higher temperature, 28° C., speeded up development and greatly decreased the in-

TABLE III

Influence of sucrose concentration on eye-color (starvation effect) and length of larval life. Basic medium: 0.5 per cent brewers' yeast in 1.5 per cent agar. (Figures in parenthesis indicate number of flies.)

Prolongation of larval life	Sucrose concentration (*per cent*)							
	0	0.05	0.1	0.3	0.5	1.0	2.0	4.0
days 1			4.0–4.5 (4)	1.5–2.0 (21)	1.0–2.5 (60)	0.3–1.5 (22)		
2	2.0–3.5 (2)	4.0 (2)	3.5 (17)	3.5 (19)	2.5–3.0 (18)	0.5–1.5 (21)	0.0–0.2 (11)	
3	2.0–3.5 (7)	3.5 (20)	3.0 (10)	3.0 (7)	2.0–3.0 (6)	0.0–1.5 (8)	0.0–0.6 (19)	
4	2.0–4.0 (17)	2.0–3.0 (5)	3.0 (5)	2.5 (2)			0.0–0.5 (5)	0.0 (2)
5	2.5–3.5 (2)	1.5 (1)					0.0–0.8 (5)	0.0 (20)
Total	2.0–4.0 (28)	1.5–4.0 (28)	3.0–4.5 (36)	1.5–3.5 (49)	1.0–3.0 (84)	0.0–1.5 (51)	0.0–0.8 (40)	0.0 (22)

tensity of the starvation effect; i.e., pigment production did not take place on yeast concentrations over 0.5 per cent. At 25° C., 3 per cent yeast or more prevented the starvation effect, while pigment appeared on all concentrations up to and including 5 per cent yeast at 17° C. This effect of temperature may be due to a differential influence on

larval activity (food intake) and on the rate of metabolic processes. The medium containing 0.5 per cent yeast was selected for standard starvation and used throughout further work. At 25° C. it consistently delayed larval development from 2 to 4 days and gave eye-color values of from 2.5 to 3.5. This is equivalent to a v^+ hormone production of 3.5 to 8.0 units per individual (Tatum and Beadle, 1938). Controls were made for each series of experiments, with similar results. These control starvation values are omitted from the tables in most cases.

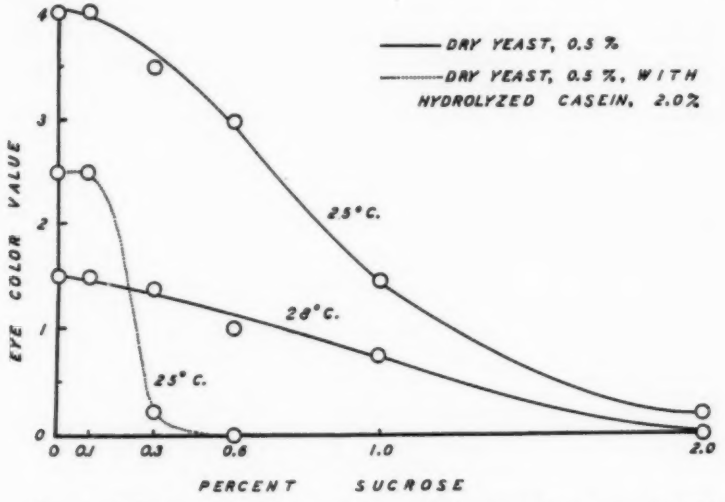

FIG. 1. Influence of temperature and of hydrolyzed casein on sucrose inhibition of the starvation effect (maximum color values for each sugar concentration used in plotting curves). Basic medium: 0.5 per cent brewers' yeast in 1.5 per cent agar.

Effect of Carbohydrates

Table II shows the influence of added carbohydrates on the starvation effect. Starch, sucrose, and glucose almost completely inhibited the production of pigment, although larval life was prolonged as much as or more than in the controls without carbohydrate. Several series of experiments were made to establish the relation of sugar concentration to prolongation of larval life and intensity of the starvation effect. Table III gives the combined results of these series. Concentrations of sucrose up to 0.5 per cent shortened larval life as compared to the control, without very marked effect on eye pigmentation. Higher sucrose concentrations progressively prolonged larval life and inhibited pig-

mentation. Two per cent sucrose caused about the same delay as in the control, but almost completely prevented v^+ hormone production. Four per cent sucrose seemed to be toxic and prolonged larval life even more than in the control, but completely suppressed the starvation effect.

The inhibiting effect of varying concentrations of sugar at 28° C. was also determined. The influence of temperature on the sucrose effect is shown in Fig. 1. It required almost the same concentration of sugar (2 per cent) to inhibit completely pigmentation at the higher temperature as at 25° C., although the production of pigment on the control starvation food without sugar was much less at 28° C.

TABLE IV

Influence of other substances on the starvation effect. Basic medium: 0.5 per cent brewers' yeast in 1.5 per cent agar.

Substance added	Prolongation of larval life in days	Number of adult flies	Eye color
Sodium benzoate, 1 per cent*	1–3	20	0.1–1.5
Sodium benzoate, 1 per cent†	2–3	5	0.5–0.8‡
Calcium acetate, 2 per cent	6–9	30	0.0–0.2
Calcium lactate, 3 per cent	5–9	30	1.0–3.0
Calcium carbonate, 2 per cent	6–9	20	2.0–3.5
Ethyl alcohol, 5 per cent§	1–5	22	0.0–0.1
Glycerol, 2 per cent	3–7	38	2.0–3.0
Butter fat, 4 per cent	3–8	11	0.0–0.2

* Sterile 60-hour-old fully fed larvae transferred aseptically to test medium.
† Eggs not sterilized.
‡ Control with no benzoate; color = 3.0.
§ Alcohol added after cooling medium to 35°C.

The results of these experiments with carbohydrates show that prolongation of larval life is not necessarily accompanied by v^+ hormone and eye pigment production. However, the starvation effect is observed only when larval life is prolonged.

Influence of Other Substances on the Starvation Effect

It seemed possible that some indication of the nature of the starvation effect might be obtained by similarly testing substances other than carbohydrates. Table IV summarizes the results of these experiments. Calcium lactate, calcium carbonate, and glycerol had only very slight inhibiting effects on pigmentation. On the other hand, ethyl alcohol, butter fat, and calcium acetate prevented the starvation effect almost completely. Sodium benzoate was quite toxic, but under non-lethal conditions it prevented pigment production to a considerable degree.

Each of these various additions to the starvation diet considerably prolonged larval life, but production of v^+ hormone was suppressed only by certain specific substances, all of which, with the exception of sodium benzoate, may be assumed to be metabolized in a manner similar to carbohydrates. No explanation can be suggested for the inability of glycerol and calcium lactate to function in this way. Concentrations of calcium lactate and glycerol from 0.5 to 3.0 per cent have been used with similar results in every concentration.

Effect of Proteins and Amino-acids

In contrast to carbohydrates, which definitely inhibit the starvation effect, whole and hydrolyzed proteins and mixtures of amino acids, including tryptophane, have no significant effect in reducing either duration of larval life or pigment production. These results are given in Table V.

TABLE V

Influence of protein and amino acids on starvation effect. Basic medium: 0.5 per cent brewers' yeast in 1.5 per cent agar.

Substance added	Prolongation of larval life in days	Number of adult flies	Eye color
Gelatine, 3 per cent	5–6	2	1.5–2.0
Gelatine, 3 per cent; Tryptophane, 1 per cent	5–9	7	1.5–2.5
Mixture of amino acids*	11	2	3.5
Hydrolyzed casein, 0.5 per cent	2–4	13	2.5–3.5
Hydrolyzed casein, 1 per cent	2–4	18	2.0–3.5
Hydrolyzed casein, 2 per cent	2–4	11	1.0–3.0
Hydrolyzed casein, 4 per cent	5–6	9	0.2–3.0

* Tryptophane, tyrosine, cystine, leucine, asparagine, glycine, alanine; 0.1 per cent each.

Although hydrolyzed casein alone had very little effect on pigmentation, it greatly intensified the sucrose effect. The result of a series containing 2 per cent hydrolyzed casein and increasing amounts of sugar is graphically represented in Fig. 1. In the presence of hydrolyzed casein, a sucrose concentration of 0.3 per cent almost completely inhibited hormone production. The other curves in Fig. 1 give for comparison the effect of sucrose without hydrolyzed casein. In the presence of an excess of amino acids, the sugar concentration effective in pigment inhibition was about that optimal for growth (see Table III).

Discussion

Khouvine et al. (1938) suggested that sugar may have a protein-sparing action, and that the starvation effect and production of v^+ hormone involves an abnormal protein degradation in the larva. It seems possible, from our results, that the action of carbohydrates and similar substances may be due to their protein-sparing action. However, it is probable that other factors are also involved since the sugar concentration optimal for growth does not inhibit the starvation effect and pigment production. This concentration (0. 5 per cent) should have the same protein-sparing action as higher concentrations. In the presence of an adequate supply of amino acids (hydrolyzed casein), however, sugar completely inhibits the starvation effect at the 0.5 per cent concentration optimal for growth.

Carbohydrates seem to inhibit pigment production in starvation by altering the starvation metabolism in such a way that v^+ hormone is not produced, and not by affecting the utilization of the hormone. Khouvine et al. showed that a diet containing sugar did not affect the utilization of ingested v^+ hormone supplied as a *Calliphora* extract. In addition, we have injected mixtures of glucose with extracts containing v^+ substance into *v bw* larvae with no decrease in the effectiveness of the hormone.

Substances other than carbohydrates which also prevent the starvation effect probably act in the same way, since theoretically they may be metabolized in a similar manner. The action of sodium benzoate, since it has no relationship to carbohydrates metabolically, may have a different basis. Sodium benzoate acts similarly to sugar in that it inhibits the production of v^+ hormone by *v bw* larvae on a starvation diet. However, it has no effect on the normal hormone production by su^2-*v, v bw* larvae (normal eye-color ♂ = 1.0; ♀ = 2.0). Nor does sodium benzoate influence the utilization of ingested v^+ hormone by *v bw* larvae.

Beadle et al. (1938) showed that starvation is effective only during a certain sensitive period in larval development. This period was found to lie between 60 and 70 hours of normal development. The starvation effect may be assumed to be a result of prolonging this specific developmental period. Preliminary experiments designed to determine the effect of sucrose on this sensitive period were carried out under aseptic conditions by placing fully fed 54-hour-old larvae on low food with and without sugar. At intervals thereafter larvae were removed from the starvation food to plain agar. The ability to pupate served as the criterion of the end of the 60–70-hour sensitive period (see Beadle et al., 1938). The results seemed to indicate that this period is significantly shortened by sugar in the starvation food.

Whether the action of carbohydrates in inhibiting the starvation effect is due to a direct influence (possibly through a protein-sparing action) on specific processes which during the starvation period lead to the production of v^+ hormone, or whether it is due to a differential acceleration of development during the 60–70-hour sensitive period, thereby shortening the effective time of starvation, cannot be definitely decided at present.

Summary

The production of v^+ eye-color hormone and development of pigment in the double recessive vermilion brown of *D. melanogaster* may be brought about by feeding the larvae on sub-optimal levels of dead yeast under aseptic conditions.

With a given concentration of yeast, culture of larvae at low temperature (17° C.) greatly increases the intensity of the starvation effect. High temperature (28° C.), on the other hand, decreases the intensity of the starvation effect.

Carbohydrates and related substances (acetate, fat, and ethyl alcohol) added to the low yeast diet, under aseptic conditions, completely inhibit the starvation effect by their direct action on larval metabolism and development.

Proteins and amino-acids have very little influence on the starvation effect, but greatly lower the carbohydrate level required to completely inhibit pigment production.

The starvation effect is always associated with prolongation of larval life, but great prolongation of life is possible under certain conditions without any modification of eye color.

The inhibition by carbohydrates may be due to a direct influence on processes proceeding during starvation or to a specific acceleration of development during the period sensitive to starvation, or to both.

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PIGMENT INHERITANCE IN THE FUNDULUS–SCOMBER HYBRID

ALICE RUSSELL

(From the Marine Biological Laboratory, Woods Hole, Mass.)

The hybrid between *Fundulus heteroclitus* (L.) ♀ and *Scomber scombrus* (L.) ♂ is first mentioned by H. H. Newman in 1915. J. Loeb had stated his belief that development in intergenic hybrids is parthenogenetic. Newman cites the inheritance of *Scomber* pigmentation in the *Fundulus-Scomber* cross as a proof that fertilization had taken place. In a later paper, 1918, he continued the discussion of this hybrid.

His account of the abnormalities found in the embryos made it seem worthwhile to obtain the cross again, to make a cytological and morphological study of early stages, and to make a detailed study of the chromatophores in parent and hybrid embryos.

Preliminary hybridizations were made successfully late in June, 1937, at the Marine Biological Laboratory. During the summers of 1938 and 1939 numerous hybridizations were made from June 10–July 5. After July 5 it is usually impossible to procure spawning mackerel, before June 10 it is difficult to obtain spawning *Fundulus*. For best results, with a large percentage of hybrid embryos, both parents must be at the height of sexual activity.

Ripe *F. heteroclitus* females were selected and isolated in tanks of running sea water for at least 18 hours before they were to be used: this assures the absence of *Fundulus* sperm. As mackerel do not live long after being caught, hybridizations were carried out at the traps. *Fundulus* females were carried to the fish traps in clean buckets or bowls of sea water. *Scomber scombrus* males were stripped into finger bowls containing a small quantity of sea water. The *F. heteroclitus* females were stripped into the sperm suspension, the eggs from each female being kept in separate bowls. After 10–15 minutes the sperm suspension was washed off and fresh sea water was added. In the laboratory the eggs were placed a few in each bowl, and allowed to develop. Controls were carried as follows: (1) Unfertilized *F. heteroclitus* eggs from some of the females in each set of hybridizations were observed as a check on the possible presence of *F. heteroclitus* sperm in sea water, in the tanks, or on the fish. (2) *F. heteroclitus* eggs were fertilized with *F. heteroclitus* sperm to check on the fertilizability and rate of develop-

ment of normal *Fundulus heteroclitus.* (3) *Scomber scombrus* eggs were fertilized with *Scomber scombrus* sperm to check on the normal *Scomber scombrus* development. (4) The reciprocal cross with *Scomber scombrus* eggs and *F. heteroclitus* sperm was tried many times, always unsuccessfully.

The egg of *Fundulus heteroclitus* is 2–2.5 mm. in diameter, well yolked, demersal, developing slowly and hatching out in 12–16 days. The egg of *Scomber scombrus* is smaller, 1 mm. or less in diameter, transparent, pelagic, developing quickly and hatching in 60–72 hours. The hybrid develops more slowly than normal *F. heteroclitus,* forming defective embryos which, in our experience, never hatch, even though kept for 30–35 days.

Normal stages of *Scomber scombrus* have not been described, although Worley (1933) mentions that they resemble closely those of sea bass as described by Wilson (1889). As there was no published account of pigment development in *Scomber scombrus,* this had to be studied. *Scomber scombrus* eggs were obtained and fertilized at the fish traps. As the eggs are pelagic, it is difficult to wash off the excess milt while in transit from traps to laboratory; sea water can, however, be added from time to time. *Scomber* eggs are very sensitive to temperatures above 17° C., and will not develop at all above 21° C. (Worley, 1933), therefore care must be taken that the water in the shallow bowls is not warmed by the sunshine, or by heat from the decks or engine. In the laboratory the fertilized and developing eggs soon float to the top of the water, and can be skimmed off and transferred to fresh sea water. If the bowls are placed in baths of running sea water the eggs develop quite normally, and hatch out in 60–72 hours. The first pigment cells to appear are the slender branching melanophores on the dorsal surface of the embryo at 27 hours. Later more melanophores appear and form the characteristic pattern; a band across the dorsal surface of the head at the level of the optic vesicles, and a row along the lateral line region. A few migrate to the yolk sac and to the oil drop. At 36 hours there appears just behind the optic vesicles a group of cells containing yellowish green pigment granules. Soon the granules increase in number, the pigment cells fuse, forming two large brilliantly green chromatophores persisting at least as long as the fry live in the laboratory. Other green chromatophores may appear behind the otic vesicles, on the oil drop or near Kupfer's vesicle. Upon hatching the young fry drop to the bottom of the vessel and lie there until they are able to swim about easily.

Normal stages of *Fundulus heteroclitus* have been described (Oppenheimer, 1937). Typical pigment formation has been described, also

(Bancroft, Stockard, Newman). Four days after fertilization a first head crop of melanophores appears. Another crop appears on the fifth day. The pigment cells are of three types: those on the yolk are large polygonal melanophores with but few processes, those on the embryo are smaller and more branched: the reddish-orange much-branched chromatophores found on embryo and yolk sac. On the sixth day, when circulation begins, the melanophores of the yolk sac migrate to the blood vessels and fuse. The reddish-orange chromatophores also arrange themselves along the course of the blood vessels, but do not seem to fuse. A number migrate to the lateral line. Melanophores are rarely seen in this region, the absence of a visible lateral line being one of the species characteristics of the adult *F. heteroclitus*. After hatching few reddish chromatophores are to be found on the exterior of *F. heteroclitus*.

The melanophores of *F. heteroclitus* and *S. scombrus*, the green chromatophores of *Scomber*, and the reddish chromatophores of *Fundulus* all contain a granular pigment. No green chromatophores are ever present in *Fundulus heteroclitus*, and no red chromatophores in *Scomber scombrus*.

Fundulus-Scomber hybrids cleave at the same rate, or more slowly, than normal *Fundulus heteroclitus*. In a series of 15 hybridizations, 3,097 eggs were fertilized: 3,084, or 99 per cent of these cleaved. Many died at gastrulation and during early embryonic life, but 1,205 or 39 per cent formed advanced embryos. Development in the hybrid is slower than in normal *F. heteroclitus:* pigmentation develops later, the heart does not begin to pulsate as early, circulation is feeble, or not established in most of the hybrids. As a result, the chromatophores remain scattered for a longer period, eventually migrating to the heart, or to the site of its attachment to the yolk. As has been noted by Bancroft, Newman and others, the hybrid embryos show various combinations of the parental types of chromatophores.

Figure 1 *a* shows the average melanophore counts for parent and hybrid embryos. As their rate of development differs widely stages which are equivalent were arbitrarily chosen. Normal *F. heteroclitus* five days after fertilization shows both first and second crops of melanophores not as yet fused on the blood vessels. *Scomber scombrus* at 30 hours shows first and second crops of melanophores also, not yet migrated to head and lateral line regions; the hybrids at 7 days showed the melanophores well developed, not yet fused. *F. heteroclitus* embryos show from 8–18 melanophores, or an average of 13—on the dorsal surface; *S. scombrus* has from 34–51, averaging 40. The *Fundulus-Scomber* hybrid shows a great variation, from 2–44. The majority of

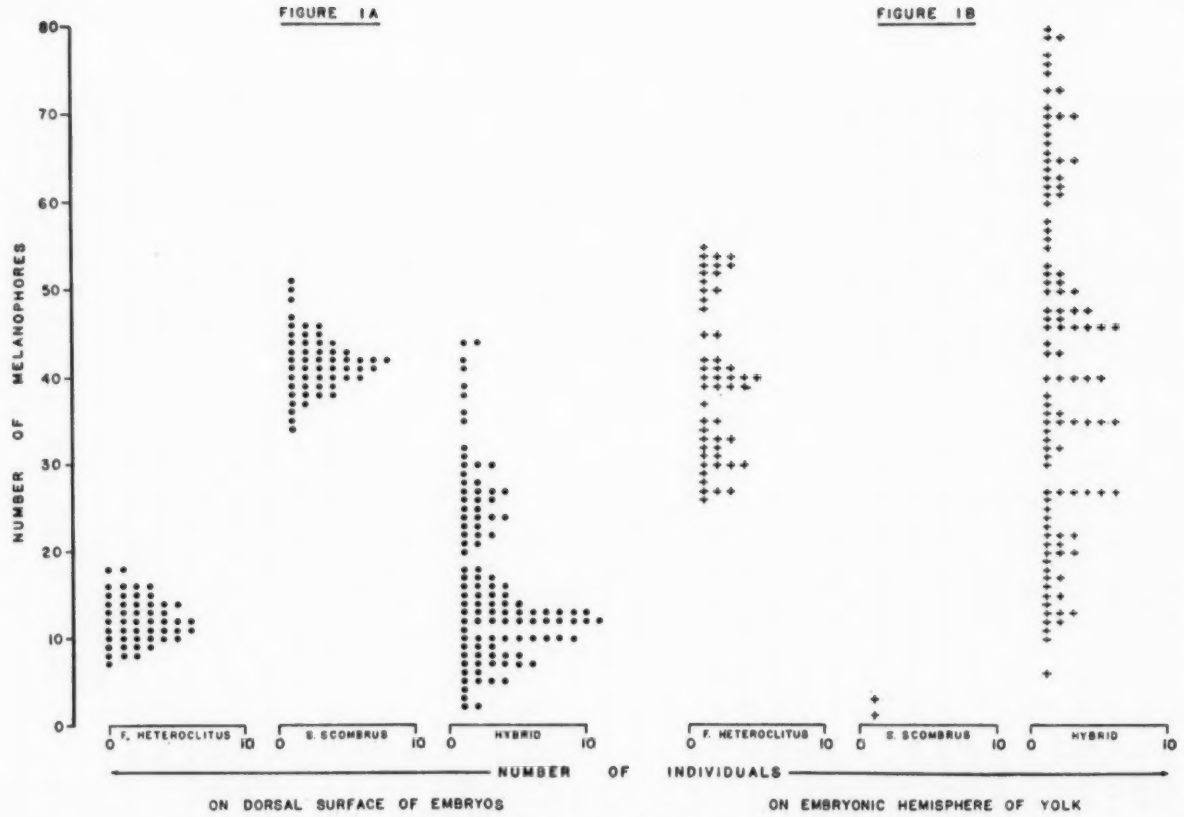

FIG. 1. *A* and *B*. Distribution of melanophores in parent and hybrid embryos.

hybrid embryos seem to show a tendency to conform to the *F. heteroclitus* type of distribution, having 8–18 melanophores on the dorsal surface of the embryos. However, we have seen no normal *F. heteroclitus* embryos of 5 days with over 20 melanophores—the increase in number of melanophores present in a large number of hybrid embryos may be assumed to be the influence of *Scomber scombrus*.

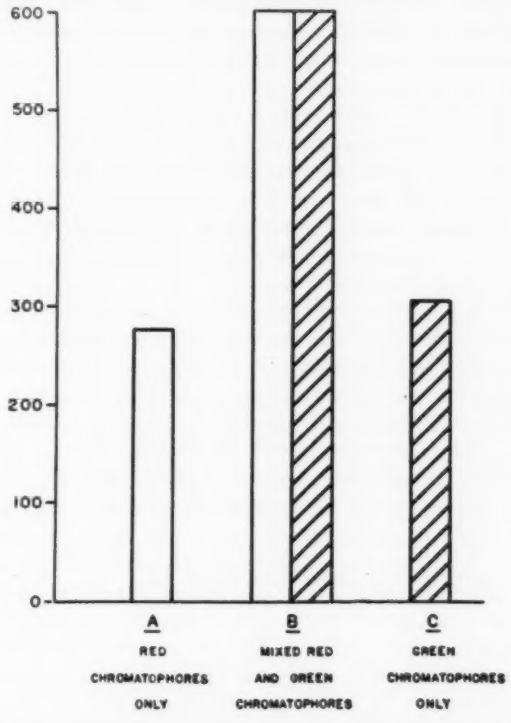

FIG. 2. Chromatophore inheritance in the hybrid embryos.

Figure 1 *b* shows the distribution of yolk melanophores, counted on the embryonic hemisphere of the same individuals used above. In *F. heteroclitus* the yolk melanophores are large polygonal cells, varying from 26–55 in 5-day-old embryos. *Scomber scombrus* at 30 hours rarely shows any yolk melanophores. In the 7-day-old hybrid embryos the number varies from 6–80, with scarcely two embryos having identical numbers of melanophores.

The hybrids can be grouped roughly into three categories: those showing green chromatophores of the *Scomber* type, but no red *F. heteroclitus* type chromatophores: those showing the red *Fundulus* type, but no green *Scomber* type chromatophores: those showing both red and green chromatophores. Figure 2 will show the distribution of 1,205 ten-day-old hybrid embryos in these three categories. There seems to be a very significant relation at first glance, since about equal numbers of individuals show only maternal or paternal type chromatophores, and about twice as many show both types combined. As a matter of fact, examination shows that no two embryos are identical. In the group showing green chromatophores only, the individuals range from those brilliantly green laterally to some with only a few green head chromatophores: some show green chromatophores on the embryo only, some show them on the yolk also. In the group showing only red chromatophores of *Fundulus* type, the individuals are equally variable, some embryos being brilliantly reddish-orange, others grading to some resembling closely *F. heteroclitus* embryos. In the large group showing both red and green chromatophores there is every conceivable type of combination, no two individuals are identical. In addition, the *Scomber* and *Fundulus* types of melanophores are present in all possible combinations with the red and green chromatophores, in all three categories. No consistency of pigment distribution is found in sets of *Fundulus* eggs from various females, fertilized at the same time by sperm from the same mackerel: some may show a preponderance of green, of red, or of mixed green and red chromatophores as will be seen in Table I.

TABLE I

Green Chromatophores Only	Mixed Red and Green	Red Only
84	57	43
27	41	3
27	107	35
2	29	21
2	12	29

These counts were made on eggs from various *F. heteroclitus* fertilized by sperm from the same mackerel on June 17, 1939, and counted June 27, 1939.

Unlike Newman, who reports that green chromatophores can be found only in hybrid embryos obtained before mid June, we have found green chromatophores in the hybrids whenever we got successful crosses.

DISCUSSION

Many intergenic and interspecific crosses have been described. Some of these "hybrids" do not develop beyond late embryological

stages, as is true of the *Fundulus-Scomber* hybrid we are discussing. Here we are dealing with a much wider cross, for the two genera belong to different sub-orders. They differ widely in ecological relations, in habitat, and in structure. Morphological studies of hybrid and parent embryos may show significant combinations of structural peculiarities, for example: *F. heteroclitus* has an air bladder, while this structure is absent in *Scomber scombrus*. Cytological studies may show successive elimination of chromosomes during early cleavages. It is probable that some of the embryos showing only *Fundulus* type chromatophores, and typical *Fundulus* distribution of melanophores, may be haploid individuals. These individuals are rare, however, for even in this group one generally finds some *Scomber* type melanophores, or some *Scomber* effect on the number or distribution of melanophores. (Figure 1, *a* and *b*.)

It is probable that in the teleosts, as in the amphibians, pigment differentiation depends on neural crest development. If so, it is reasonable to suppose that wherever *Scomber* type chromatophores are present, *Scomber* chromosomes may have been retained throughout cleavage, gastrulation and differentiation.

We have purposely refrained from a discussion of the size of melanophores and chromatophores in parents and hybrids, for any measurements would be open to the criticism that metabolic processes in the embryo are abnormal, or at least, much disturbed. In shape, the melanophores of *Fundulus* are quite distinct and distinguishable (Newman and others). In color the reddish chromatophores in the hybrids are identical with those of *Fundulus heteroclitus*, the green chromatophores identical with those of the *Scomber* parent.

In the literature on interspecific and intergenic crosses, the F_1 generation is generally reported as intermediate. A closer scrutiny of the hybrids may reveal a much wider variation than hitherto suspected, as, for instance, in the case of the melanotic hybrids between *Platypoecilius* ♀ and *Xiphophorus* ♂ described by Gordon. Apparently a case of Mendelian dominance is revealed in the universally melanotic F_1 generation, but there is reported a variation in degree of melanosis in the progeny.

Earlier reports (Newman, Bancroft) have attempted to explain the results of the *Fundulus-Scomber* hybridizations on the basis of Mendelian dominants and recessives, or of "blending" inheritance. However, as no detailed study of pigmentation was attempted, the enormous variability actually present in the hybrids escaped attention.

Pinney, reporting on other inter-sub-order hybrids, reports chromosome elimination during early cleavages.

We have at this time no satisfactory explanation for the phenomenon presented by the pigment inheritance in this cross, although we may assume that there is a complicated random assortment, combination and elimination of chromosomes.

We are indebted to Mr. Robert Goffin of the U. S. Bureau of Fisheries, at Woods Hole, and to the crew of the "Sagitta" of the Marine Biological Laboratory for their assistance in getting to the fish traps and obtaining mackerel. We wish also to thank Dr. H. B. Goodrich, for his interest, and also Rev. F. W. Ludwig, Ph.D., for help with the microphotographs.

Summary

1. Methods for hybridizing *Fundulus heteroclitus* and *Scomber scombrus* are described.

2. The pigment development in hybrid and parents is described.

3. Comparison of the inheritance of embryo and yolk melanophores reveals a *Scomber*-effect in the embryo.

4. The hybrid embryo shows chromatophore inheritance from both parents.

5. Actually, as regards inheritance of melanophores and chromatophores, there is enormous variability, no two embryos being identical as to pigment distribution.

6. This variation in the F_1 generations is unusual and at present inexplicable.

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THE EFFECT OF INCREASING TIME OF DEVELOPMENT AT CONSTANT TEMPERATURE ON THE WING SIZE OF VESTIGIAL OF DROSOPHILA MELANOGASTER

GEORGE CHILD

(From the Department of Biology, Amherst College, Amherst, Massachusetts)

Introduction

This paper deals with the effect of nipagin (methyl parahydroxy benzoate) and poor food conditions on the wing size of the mutant vestigial in *D. melanogaster*. Nipagin is being used in many laboratories as an antiseptic for mold control in *Drosophila* culture media. It was found at this laboratory that the time of development of an isogenic stock was increased when the larvae were raised on nipagin-treated food. This suggested a method for increasing the time of development at constant temperature, a new tool in phenogenetic research.

The effect of temperature on the wing size of vestigial has been studied by a number of investigators (Harnly, 1930, 1932; Stanley, 1928, 1931, 1935; Hersh and Ward, 1932; Li and Tsui, 1936). They find that the wing size of vestigial increases with increasing temperature. Furthermore, the temperature-effective period occurs during a relatively short portion of the larval life. This present work represents a different approach to the problem in that it concerns the effect of prolonging the duration of the larval period at constant temperature.

Methods

The culture medium consisted of 850 cc. water, 100 grams cornmeal, 150 cc. molasses, 13 grams agar-agar, and 6 grams brewer's yeast, made up in the usual manner. The cultures were seeded with dry yeast. In the starvation experiments the dry yeast was not added. The nipagin was weighed on a standard quantitative balance and thoroughly mixed with the food before pouring. Half-pint milk bottles containing 60 cc. food were used.

The isogenic vestigial stock was obtained from Dr. A. Hersh of Western Reserve. About 20 pairs were used for egg-laying. It was found that the flies did not lay well on the food containing the higher concentrations of nipagin. It was necessary to use long egg-laying periods of twelve hours and for this reason the time of development was determined only approximately for these concentrations.

The egg-laying and total development was carried out in an incubator held constant at 28° ± 0.1°. The incubator was kept in a constant temperature (16° ± 1.°), constant humidity (60 per cent ± 5 per cent relative humidity) room. The apparatus is fully described in Drosophila Information Service 6, April 1936.

As the flies hatched they were examined, the sexes were separated and they were placed in vials containing 70 per cent alcohol. An unselected sample of control flies and 0.2 per cent nipagin flies were bred for a second generation to determine any "carry-over" effect. The wings of the flies were removed under a binocular microscope and

TABLE I

Effect of nipagin on wing size of vestigial. Temperature, 28° ±0.1°.

Conc. of Nipagin in per cent	Time of Pupation (hours)	♀♀			♂♂		
		No.	Length in mm. ±s.e.	Area in mm.² ±s.e.	No.	Length in mm. ±s.e.	Area in mm.² ±s.e.
0.0	98	40	0.85 ±0.017	0.178 ±0.0047	14	0.76 ±0.023	0.132 ±0.0033
0.05	104	44	0.90 ±0.007	0.186 ±0.0043	25	0.91 ±0.035	0.177 ±0.0078
0.1	112	37	0.90 ±0.015	0.171 ±0.0044	41	0.95 ±0.024	0.189 ±0.0072
0.2	146	50	1.02 ±0.003	0.213 ±0.0094	45	1.06 ±0.035	0.221 ±0.0094
Carry-over effect							
F_1 from random sample of .2 per cent nipagin-treated flies							
0.00	103	31	0.95 ±0.023	0.206 ±0.0055	38	0.85 ±0.0075	0.167 ±0.0038

mounted on slides with a drop of cedar oil. The right wing was used unless it was torn or mutilated.

The wings were projected with a Proni projection apparatus. The magnification set at 75 × was checked periodically with a stage micrometer. The periphery of the wings were traced and from these tracings the maximum lengths and areas were measured. A Glogau vernier caliper and a Keuffel and Esser planimeter were used.

In the later experiments 0.1 per cent nipagin was used. It was suspected that nipagin produced its effect by slowing down the growth of yeast and thereby decreasing the food supply. To check this a number of cultures were prepared with no addition of live yeast. In these non-

seeded bottles a number of old larvae were added after the egg-laying. This was done to remove any yeast carried in on the bodies of the adult flies.

The time of development was determined by removing pupae at intervals of four hours and placing them on agar slants. Many of these flies were used for a second generation test. The matings were control × control, control ♂♂ × nipagin-treated ♀♀, control ♀♀ × nipagin-treated ♂♂ etc. to determine how much of the carry-over effect was maternal or paternal.

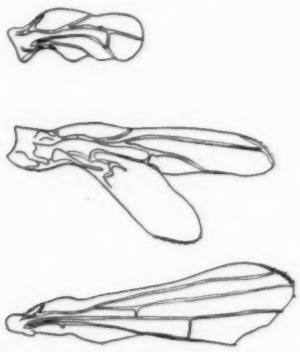

FIG. 1. A normal 28° vestigial wing compared with wings from 28° nipagin treated flies.

EXPERIMENTAL

The Effect of Varying Concentrations of Nipagin

In these experiments the larvae were raised on nipagin-treated food to determine the effect of nipagin on the time of development and wing area. The concentrations of nipagin used were 0.05 per cent, 0.1 per cent, 0.2 per cent, 0.4 per cent, and 0.8 per cent of the food weight. A few larvae in the 0.4 per cent and 0.8 per cent developed to pupation but failed to hatch. It was found that the time of development increased with increasing concentrations of nipagin, the 0.2 per cent pupating two days later than the controls.

Table I shows the wing length and area as affected by nipagin. With one exception, the 0.1 per cent for females, the lengths and areas of the vestigial wings increase with increasing concentrations of nipagin. The wing size of the control males is smaller than that of the females. With increasing concentrations of nipagin, however, the male wing size increases faster than the female. At 0.1 per cent and 0.2 per cent the

male wings are larger than the female. A similar result is obtained with temperature, the male wing size exceeding that of the females at high temperature (Harnly, 1930; Stanley, 1931).

It was found when preserving the flies at hatching, that with increasing time of development there was an apparent increase in wing size within each nipagin-treated population. The change in wing size due to nipagin is, therefore, greater than the means given in Table I; these means having been obtained by including all the flies in a given population irrespective of their time of development. This general result has been recently reported by Braun (1939) on notch.

TABLE II

Relation between time of pupation and wing area. Control series.

Time of Pupation in hours		♂♂		♀♀	
		No.	Area in mm.	No.	Area in mm.
1.	82	8	0.130	3	0.164
2.	86	6	0.138	6	0.137
3.	90	15	0.137	6	0.159
4.	94	25	0.136	10	0.143
5.	98	7	0.154	11	0.173
6.	102	3	0.158	4	0.147
7.	106	3	0.133	2	0.187
8.	110	0		2	0.167
9.	114	3	0.107	2	0.154
mean time, 93.2±0.87 hours mean area, 0.139±0.004 sq. mm.				mean time, 96.1±1.24 hours mean area, 0.156±0.0035 sq. mm.	

Figure 1 illustrates the appearance of the larger wings obtained from a 0.2 per cent nipagin population as compared with a "normal" vestigial wing. The larger wings simulate the expression of other vg. alleles when raised under normal environmental conditions.

The results obtained by raising a random sample of 0.2 per cent nipagin-treated flies for another generation but in untreated food are shown in Table I. Both the mean length and area show a significant carry-over effect. Some of these data had been reported previously (Child and Albertowicz, 1936).

Effect of Time of Development

In the second series of experiments the relation between time of development and wing area was determined. Starvation and 0.1 per cent nipagin were used. The larvae were removed from the culture as

they pupated and the areas of the wings were determined separately for each pupating group. The results (Table II) indicate that in the control series there is no apparent effect of time of development (from egg-laying to pupation) on the size of the wings. The larvae pupate between 82 hours and 114 hours and the wing areas among the different groups do not differ significantly from one another.

The time of development is very markedly increased in the nipagin and starvation series (Tables III and IV). The wing areas of the various groups show greater differences than in the control series. There is an apparent relation between the time of pupation and wing

TABLE III

Relation between time of pupation and wing area after treatment with 0.1 per cent nipagin.

Time of Pupation in hours	♂♂		♀♀	
	No.	Area in mm.	No.	Area in mm.
1. 93	10	0.158	12	0.167
2. 97	8	0.231	5	0.181
3. 101	2	0.437	4	0.140
4. 107	23	0.299	12	0.172
5. 118	9	0.368	8	0.181
6. 129	5	0.592	5	0.236
7. 141	6	0.517	1	0.268
8. 153	1	0.505	3	0.233
9. 165	25	0.268	8	0.151
10. 179	23	0.267	23	0.165
11. 191	7	0.318	14	0.208
12. 203	12	0.379	14	0.190
13. 215	14	0.354	27	0.201
	mean time, 152.4±3.39 hours mean area, 0.315±0.0113 sq. mm.		mean time, 163.5±3.81 hours mean area, 0.185±0.0044 sq. mm.	

area. This relation is more easily observed on the imagoes as they hatch. With increasing time of development the larvae (and the flies) become smaller and smaller so that the relative difference between wing area and body size is very great in the delayed flies although the absolute area increases and then decreases. Unfortunately the body size of the adults was not measured and we are unable to show this difference quantitatively.

The Carry-over Effect

The carry-over effect was studied using normal food. To determine whether both sperm and eggs from treated parents transmitted the fac-

TABLE IV

Relation between time of pupation and wing area after starvation.

Time of Pupation in hours	♂♂		♀♀	
	No.	Area in mm.	No.	Area in mm.
90	7	.204	5	0.190
94	3	.164	3	.310
98	2	.197	12	.185
102	5	.194	2	.238
106	1	.224	4	.188
110	2	.169	7	.169
116	17	.277	16	.201
122	4	.258	8	.196
126	4	.283	4	.215
130	3	.212	4	.100
138	6	.242	9	.300
150	0		3	.231
162	1	.345	1	.212
174	2	.642		
	mean time, 116.4±2.53 hours mean area, 0.254±0.0174 sq. mm.		mean time, 115.0±2.13 hours mean area, 0.213±0.0102 sq. mm.	

tors for increased wing size, treated males and females were mated with control females and males respectively. Treated males were also mated with treated females. The results of these various reciprocal crosses are shown in Table V which also includes the control areas and the means of Tables III and IV. The carry-over effects are more apparent

TABLE V

Effect of .1 per cent nipagin and starvation.

Experiment	♂♂			♀♀		
	Time of Pupation	No.	Area ±s.e.	Time of Pupation	No.	Area ±s.e.
	hours		*mm.²*	*hours*		*mm.²*
Control	93.2±0.87	70	0.139±.0040	96.1±1.24	48	0.156±.0035
.1% Nipagin	152.4±3.39	145	0.315±.0113	163.5±3.81	136	0.185±.0044
Starved	116.4±2.53	57	0.254±.0174	115.0±2.13	78	0.213±.0102
Previous Treatment	Carry-over effect F_1 from control and treated flies					
Control ♀ × Nipagin ♂	110	94	0.203±.0058	110	81	0.181±.0019
Control ♀ × Starved ♂	110	33	0.203±.0163	110	49	0.183±.0025
Nipagin ♀ × Control ♂	116	25	0.229±.0270	116	36	0.201±.0167
Starved ♀ × Control ♂	116	45	0.281±.0203	116	56	0.192±.0060
Nipagin ♀ × Nipagin ♂	116	40	0.279±.0215	116	44	0.174±.0042
Starved ♀ × Nipagin ♂	116	55	0.356±.0259	116	31	0.224±.0141
Control ♀ × Control ♂	95	22	0.143±.0052	95	19	0.161±.0037

in the male offspring than in the female offspring, since in the latter the total effect is smaller. The time of development is only approximate, not having been measured by pupa removal but by simply noting the time when about half the larvae had pupated. It is quite evident that in all of the matings the wing areas are greater when affected flies of either sex are used as parents. When treated females are used as parents the difference is greater than when males are used. Treated males and females as parents have offspring with greater wing areas than those obtained when only one treated parent is used. Starvation of the parents seems to produce a greater effect in the offspring than nipagin treatment.

Discussion

It is well known that with increasing temperature there is an increase in the wing size of vestigial. A sharp increase is not obtained, however, until very high temperatures are reached. It is generally accepted that temperature produces its effect by affecting differentially the rate or duration of the " vestigial reaction " as compared with the rate or duration of other developmental processes. By vestigial reaction we mean the developmental reaction or reactions in the vestigial fly which differ in rate or duration from the reactions in their isogenic wild type.

In the experiments with nipagin there is little reason to suspect that the change in wing size is due to a direct effect of nipagin on the vestigial reaction. The evidence, moreover, indicates that nipagin produces its effect by increasing the time of development. The temperature-effective period of the vestigial reaction is known to occur during a portion of the larval development. Thus, by increasing the larval period at constant temperature an effect on the vestigial wings will be produced if the duration of the vestigial reaction as compared with the rest of development is differentially affected. It appears from these results that such is the case.

In the first experiments, using varying concentrations of nipagin, it appeared that this chemical increased the time of development by decreasing the food supply. The yeast did not grow very well in the treated food although all bottles started with equal amounts of dead brewer's yeast and live yeast. The starvation experiments showed that this was the case. There was a definite increase in the time of development in wing area under both types of environmental conditions.

As stated previously, the exact relation between time of development and wing area is obscured because of the decrease in the size of the fly with increasing time of development. Under normal conditions the larvae begin to pupate at 82 hours and the last larvae pupate at 114

hours in these experiments. This variation is great because of the four-hour egg-laying periods but with even shorter egg-laying periods a spread of 18–24 hours is obtained (Powsner, 1935; Child, 1935).

This variation is a direct corollary to the nature of development which as Wright (1934) points out is the result of a large number of physical and chemical reactions, the rates and durations of which are determined by the history of the organism prior to the stage in question, correlative reactions within the organism, external environmental factors, actions of the genes within each cell, etc. In the highly heterogeneous systems of a developing larva these reactions will not go on at exactly the same rates in all organisms and there will of necessity be a spread in time of development as well as wing area under normal conditions *but no correlation between these measurements.* However, when an additional factor, lack of food, is superimposed upon this normal variation a new set of conditions prevails. The duration of the larval (feeding stage) period is lengthened, various reactions may produce minimal or even subminimal concentrations for further development and development will become somewhat disorganized. In other words, there will be a differential effect on the rates and durations of many embryological processes resulting in a modified phenotype. Under such conditions there will be a definite correlation between time of development and wing area.

With this general hypothesis in mind it is possible to postulate a number of mechanisms to account for the increased wing size. A simple scheme would allow the wing formation reactions to proceed at their normal rate but the developmental reactions which normally parallel them are slowed down, especially those reactions which determine the time at which the wing development stops. This would permit of an increased wing area. With further starvation even the wing reactions are slowed down or produce subminimal concentrations and the size of the wing decreases. This outline is, of course, very general and is not the only one which can be postulated. It merely illustrates how the general theory can be utilized.

Carry-over Effect

The carry-over effect experiments were unfortunately not extended beyond the first generation. They show, however, that there is a definite effect on the offspring of parents raised under poor food conditions—a sort of dauermodification (Jollos, 1934). It is well known that starved flies lay smaller eggs than normal ones. Powsner (1935) found that eggs laid by flies raised on poor food had a longer developmental period than eggs laid by flies raised on good food. If this delay

in development were the only factor involved a definite carry-over effect should be expected on the maternal side. In these experiments, however, there was also a paternal effect. To account for this result one must assume an effect of starvation on developing sperm. This may concern the small amount of cytoplasm carried by the sperm or perhaps a direct effect on its genic material.

The recent series of investigations at Columbia University by Rittenberg, Schoenheimer, Clarke and others in which deuterium, isotopes of nitrogen, and other elements were used to follow intermediary metabolism may bear on this problem. These workers have shown that many of the organic substances in protoplasm, even proteins, are not in a static condition. The "living proteins" are constantly interacting with their environment and may exchange their hydrogen for deuterium, and even nitrogen for one of its isotopes. Thus the composition and behavior of protoplasm is directly modified by the composition of its environment. Should the chromosomes or the genes behave in this kinetic manner of extracting substrates from the cytoplasm and releasing equivalent substances in exchange, we would have a mechanism for the production of these starvation effects and other dauermodifications, production of immunity, even differentiation during ontogeny. It is necessary, of course, to assume that the cytoplasm of the treated flies differs from that of normal cytoplasm. In this manner a modified cytoplasm may produce a change in the chromosomes. It is also of interest to note that if this is the case, we have a mechanism for an "inheritance of acquired characters," not in the old sense of the phrase but on a molecular level. This would allow the environment to produce "genetic changes" which need not be permanent. These "mutations" could return to normal in one or more generations. Plough and Ives (1935) found that variations continued to appear in generations later than those actually treated with a high temperature of 36.5° for 24 hours. These variations decreased in number in subsequent generations.

These experiments are to be continued for a number of generations. The original vestigial stock used has been discontinued in this laboratory and another isogenic stock is being prepared.

Summary

The time of development of an isogenic vestigial stock of *D. melanogaster* was increased by two methods: (1) by adding nipagin (ethyl parahydroxy benzoate) to the food, and (2) by adding only very little yeast to the food. Both methods are essentially the same in that the developing larvae are under starvation conditions. With increasing

concentration 0.05, 0.1, 0.2 and 0.4 per cent, there was an increase in the time of development and increase in the size of the wings, males showing a greater effect than females. The large wings resembled those of other vestigial alleles raised under normal conditions.

In another series of experiments 0.1 per cent nipagin and starvation were used. The larvae were removed from the culture as they pupated, to determine the relation between time of pupation and wing size. The first flies to pupate did not differ significantly in wing size from controls at that temperature. With increasing time of development there was an increase in wing size. Larvae which were very much delayed, however, developed into small flies with small wings. These wings, although small, were more differentiated and larger than the control wings.

The "carry-over" effect was studied using normal food. The treated females and males were mated with control males and females respectively. Treated males were also mated with treated females. The wings of flies from the latter mating showed the greatest carry-over effect. Treated females by control males resulted in flies having a significantly larger wing size than flies from the reciprocal cross. These results indicate that there is a definite effect on the germ cells of flies raised under starvation conditions, which effect shows itself in the subsequent development of the zygote.

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THE METHOD OF FEEDING OF TUNICATES

G. E. MacGINITIE

(From the William G. Kerckhoff Marine Laboratory of the California Institute of Technology)

Introduction

This, the second of a series of papers (MacGinitie, 1939) on the feeding mechanisms of marine invertebrates, deals with the method of feeding in three tunicates, namely, *Ciona intestinalis* (Linn.), *Ascidia californica* Ritter and Forsyth, and *Diplosoma pizoni* Ritter and Forsyth. The first two are simple ascidians and the latter is a colonial form. Since both simple and colonial forms have been investigated, I feel that it is fairly safe to state that the method described below is typical of all ascidians.

Young specimens of *Ciona intestinalis* and of *Ascidia californica*, especially those which have been reared in the laboratory, are quite transparent, and the observations here recorded were made upon animals which were in no way disturbed while they were carrying on their natural feeding activities. The same can be said of *Diplosoma pizoni*, as the matrix of the colony is perfectly clear and transparent. All observations were made upon undisturbed individuals of the colony.

Mechanism for Feeding

The structures which are strictly connected with the feeding activities of tunicates are the endostyle with its mucous glands, the peripharyngeal grooves, the dorsal groove, the stigmata, the esophagus, and the cilia lining all grooves, bars and the inner edges of the stigmata. The pharynx or branchial basket has been too well described in textbooks of zoology to make it necessary to redescribe it here. A current of water is maintained through the branchial cavity almost continually, whether the animal is feeding or not. The only time that the current is stopped is when the animals are left exposed by the tide or when they have been disturbed by some outside stimulus, and at such times the oral aperture and atriopore are usually closed.

The cilia lining the stigmata and the branchial basket may be divided into two groups, each group having a particular function. Those lining the stigmata have the function of maintaining the current of water, while those on the inner surface of the branchial bars and in the endo-

style, the peripharyngeal grooves and dorsal groove have the function of moving mucus.

Food and the Method of Feeding

The endostyle is richly supplied with mucous glands, and when a tunicate starts to feed it begins to secrete mucus throughout the length of the endostyle. This mucus is moved by the cilia of the branchial bars around the branchial basket in two sheets, one on either side. When the edges of the mucous sheets arrive at the dorsal groove, they are taken up by it and formed into a thread, and this string is passed posteriorly along the dorsal groove to the esophagus. The function of the peripharyngeal grooves is to hold and move the oral ends of the two mucous sheets.

The water entering the branchial basket through the oral funnel passes into the atrial cavity through the stigmata in all directions with the exception of the region of the endostyle and dorsal groove, and when the animal is feeding such water must also pass through the sheet of mucus which covers the interior of the basket. This mucus intercepts and entangles all solid material entering with the water, and such material comprises the food of tunicates. On rocky shores it consists almost entirely of plankton, often greatly enriched by algal spores from seaweeds. Within the estuaries it consists largely of material in suspension, mainly stirred-up detritus from the shores and bottom. During the summer season in Southern California this detritus in suspension is usually enriched by one or more species of dinoflagellates.

While a tunicate is feeding mucus is constantly being secreted, and the mucous sheets covering the inner walls of the branchial basket move continuously from the endostyle toward the dorsal groove. Hence, while the tunicate is feeding, the food-laden thread of mucus enters the esophagus in an unbroken string. As it enters the stomach this mucous string is folded back and forth and remains intact for some time. It is only that portion near the pyloric valve that coalesces and becomes semi-liquid as it passes into the intestine.

Although the cilia of the stigmata and branchial basket beat almost continuously, the mucous sheets are formed discontinuously. Upon the least disturbance the animals will cut off the secretion at the endostyle, and the remnants of the mucous sheets will continue to pass around to the dorsal side until the ends reach the dorsal groove. From then on until the animal begins to feed again the branchial basket is practically free of mucus. When a tunicate is not feeding, small particles may be seen to pass readily through the stigmata into the atrium and out with the atrial current.

If material which is foreign to the usual run of food material is introduced into the current of water entering the oral funnel, feeding will cease at once, and the undesirable material will be quickly forced from the branchial basket by a quick contraction of the body wall. The current will be renewed immediately, and, if no further undesirable material is taken in, feeding will soon be resumed. If the stimulus from the introduced material is rather strong the animal will cease feeding and will forcibly eject what water is in the branchial basket and atrium, and will remain closed for a considerable length of time, depending upon the strength of the stimulus.

In tunicates there is a ring of tentacles which interlace across the oral funnel which prevents the entrance of large particles. Such large particles as do find their way into the branchial basket are not incorporated in the mucus, but are in some way dropped from it into the branchial basket, and at intervals are forcibly ejected from the oral funnel by a sudden contraction of the body wall of the tunicate.

It is characteristic of animals which use mucus to entrap their food that they are able to drop from such mucus at least a portion of the undesirable material which is entrapped. Just how this is accomplished is not at present clear. It may be that the cilia which move the mucus can, by pressing outward through the mucus cause such particles to drop out. Many animals have specialized regions where the cilia perform this function. In the tunicates it is the cilia bordering the dorsal groove, in the pelecypods (future paper) it is the cilia of the lower edge of the gills and those of the labial palps, and in the echiuroid *Urechis* it is the outer cilia bordering the proboscis. In such regions the cilia are usually considerably larger than elsewhere.

When large particles strike the tentacles of the oral funnel they are usually blown away by a quick contraction of the mantle wall with little cessation of the feeding current. As most single tunicates hang vertically with the osteum downward one ejection movement serves to remove the large object. But, because of the separate action of individuals of a colony, a particle upon the surface of a colonial form which is fairly level may be bounced over the surface for some time before it is carried away by currents or is rolled over the edge. Since there is a constant current out of the atriopore (which in colonial forms may be common to several individuals), no particles find their way into the atrium and no tentacles are necessary, for if the current is stopped the atriopore closes.

As has been stated above, the cilia beat almost continually, and normally when the tunicate is not feeding most of the solid particles pass through the stigmata and out the atriopore. However, even when the

branchial basket is not lined with the mucous sheets, some of the solid particles may find lodgment upon the ciliary tracts lining the branchial basket (particularly those of the endostyle, peripharyngeal grooves and dorsal groove), and will follow more or less the definite tracts. This is especially true of specimens that are handled or cut open, since they may secrete mucus along these grooves, whereas normally they would not do so. The mucus which carries such particles as are transported along these ciliary tracts may enter the esophagus or may be dropped into the branchial basket and be ejected through the oral funnel. The more or less abnormal performance just described has led to the erroneous ideas found in textbooks about the feeding of tunicates.

Ciliary Action

The ciliary action of *Ascidia californica* was studied in detail. The oral aperture, the atriopore, the cilia of the basket, and the cilia of the stigmata may all function independently of each other or they may all function together. The cilia of the stigmata may be stopped without stopping those of the basket. When the cilia of the stigmata cease vibrating they lie down against the edges of the openings, leaving the stigmata wide open. However, when the animal is contracted the edges of the stigmata are approximated and the openings closed. At such times, of course, the cilia are still and lie flat against the sides of the openings.

After the cilia have been stopped they resume their beating by starting to vibrate in a small circle at the center of the stigmatal opening, and this ring spreads towards either end of the opening until all are again beating. The beating cilia surrounding a stigmata remind one of an elongated wheel organ of a rotifer or a veliger larva. In the ascidian investigated the apparent movement was in an anti-clockwise direction as viewed from the outside.

There is no doubt that the cilia of the branchial bars, ridges and grooves actually hold and move mucus. The cilia seem partially to enter the sheet of mucus and force it forward. During part of the beat the cilia are more or less hooked into the mucus and this serves to hold it so that the cilia following are able to penetrate and in turn do their share of pushing and holding. This action of the cilia is further evidenced by the fact that the mucous sheet which is present on the inside of the basket when the animal is feeding has in it waves which correspond to the wave motion of the cilia. These waves in the mucus appear when water heavily laden with food is introduced into the oral funnel. As the food material collects in the mucous sheet it sometimes

appears in streaks which are more accentuated as the mucus nears the dorsal groove.

Summary

1. The feeding method of *Ciona intestinalis* and *Ascidia californica* (simple ascidians), and of *Diplosoma pizoni* (a colonial form) was investigated.

2. Tunicates feed by straining the solid material from a current of water as it passes through a thin film of mucus lining the branchial basket.

3. The mucus is constantly secreted at the endostyle and is continually moved to the dorsal groove in two sheets which line the interior of the basket. The dorsal groove forms the edges of the food-laden sheets into a thread which is passed posteriorly to the esophagus and enters it in an unbroken string. The peripharyngeal grooves serve to hold the anterior ends of the mucous sheets and move them around to the dorsal groove.

4. When a tunicate is not feeding, the inside of the branchial basket is not lined with mucus, and the solid materials pass out with the atrial current.

5. Some sorting is carried out by the cilia of the dorsal ridges. The cilia which line the openings of the stigmata, and whose vibration creates the current of water passing through the basket, may be stopped without stopping the cilia lining the basket or without closure of the oral aperture and atriopore.

6. After the cilia lining the stigmata have been stopped they commence to beat in what appears to be a ring at the center of the opening. The cilia of one side of a stigmata are in perfect synchronism with those of the opposite side of the opening, and, by the continual inclusion of other cilia, all finally vibrate and resemble somewhat an elongated wheel organ of a rotifer.

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TEST SECRETION IN TWO SPECIES OF FOLLICULINA

VIRGINIA C. DEWEY

(From the Arnold Biological Laboratory, Brown University and The Marine Biological Laboratory, Woods Hole, Massachusetts)

INTRODUCTION

The fact that *Folliculina* passes through a free-swimming stage at some point in its life cycle was recognized by Wright (1859) and by Claparède and Lachmann (1858–61). The former author observed the transformation of the free-swimmer of *F. producta* into the adult form, but did not discover the mode of origin of the "larva" nor any details of the metamorphosis. For this reason these observations were later questioned by Stein (1867), but they are completely confirmed by more recent work. That the larvae are formed as the result of cell division was pointed out by Möbius (1887), but the presence of a larva and an adult lying side by side in a single test he interpreted as a case of longitudinal division. In his figures he even shows an "umbilical cord" projecting laterally from the larva. *Folliculinas* do not, however, depart from the general rule of transverse division in ciliates as was pointed out by Sahrhage (1917). Andrews (1920) has shown that free-swimming larvae may also result from the dedifferentiation of an adult form.

Test secretion and metamorphosis are described by Penard (1919) for *F. boltoni,* a freshwater species. Too few details are given to decide whether or not this species passes through the same stages as are described for *F. producta* by Andrews (1923) or for *F. simplex* (previously called *F. ampulla*) by Fauré-Fremiet (1932). Observations on *F. aculeata* and *F. elegans* indicate that the process of test formation is somewhat different in these species. During the summer of 1936 at Woods Hole, while examining fresh preparations for specimens of *Folliculina,* a form was discovered of which no description could be found. The posterior part of the body resembled that of a *Folliculina* seen on the slide, but the anterior portion was drawn out into one long, slender, flexible process bearing membranelles only at the end. Circumstantial evidence pointed to the conclusion that these forms represented a stage in the life history of *Folliculina.* Definite proof of this theory was lacking, since the larvae did not remain in good condition long enough for complete transformation to take place. This stage has evi-

dently been noted by Fauré-Fremiet (1936), but his descriptions are not detailed. In order to obtain more complete evidence concerning the significance of this stage in the life cycle, observations were resumed in 1939. A complete life history can now be given placing this stage in its proper sequence.

The classification of the members of the family Folliculinidae is still in a somewhat unsatisfactory state in spite of the fact that a number of investigators have given the matter a great deal of attention. The separation of the genus *Folliculina* into a large number of species on the basis of characteristics which are subject to considerable variation seems to be the rule. For this reason the classification given by Fauré-Fremiet (1936), in which several species which have a number of common characteristics are combined into a single species, is to be preferred. Two species, as described by this author, *F. aculeata* and *F. elegans,* seem to prevail at Woods Hole. The two are alike in many ways; the chief differences are in size, the presence or absence of pointed tips on the peristomeal lobes and in the pigmentation of the animal and of the test. Since all of these traits are subject to variation, so that one species may resemble the other very closely, it was difficult to determine which species was under observation. Certain organisms, however, presented all the criteria of one species or the other. Since the greater number of individuals observed were of a paler color and possessed the pointed peristomeal lobes characteristic of *F. aculeata,* this species is figured. The only point in which they differed from the descriptions of Fauré-Fremiet was in the color of the test which was often colorless or faintly blue. Both species have an ovoid nucleus, but, as in *F. boltoni,* it is often notched or bi-lobed.

Methods

The organisms were collected by placing glass slides in crystallizing dishes containing quantities of the hydroid *Tubularia* and leaving them for several days in running sea water. The slides were then removed and placed in Petri dishes containing sea water. Observations were made with a binocular dissecting microscope, with the 16 mm. objective of a compound microscope or with a water immersion lens (40 ×). In order to maintain the organisms in good condition over periods of several hours, a stream of fresh sea water was run into the dish while on the stage of the microscope. The overflow was carried off by means of an inverted siphon. Temperature readings were regularly recorded and during the time that the observations were being made the temperature of the sea water varied from 21° C. to 24° C.

Observations

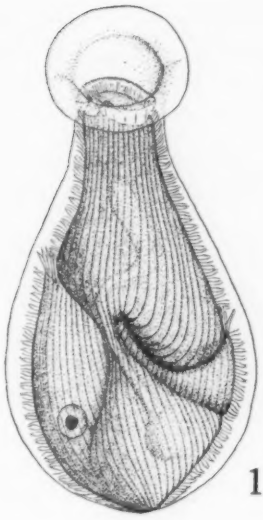
The complete life history of a larva resulting from cell division, from the time of departure from the test containing the sister cell until the adult form was attained, was followed for a number of individuals. In some cases free-swimming larvae were found and their subsequent history observed. In a few cases it was found that adult forms dedifferentiated into the larval form, left the test and settled down to secrete a new test.

After cell division (Fig. 1) the anterior individual, which will eventually leave the test as a free-swimming larva, remains for from 30 to 60 minutes in the test contracting and extending beside its sister cell, which remains attached and which has already begun the metamorphosis into the adult form. At each extension of the larva the anterior end is projected farther and farther out of the neck of the test until finally the whole organism is free. The swimming stage (Fig. 2) may last for from 15 to 90 minutes, during which time the larva swims slowly along the substratum or more rapidly near the surface of the water in the dish. At intervals it pauses to contract and extend itself at one spot and then swims on. Just before the larva settles down to secrete its test it may be seen to repeat this process of contraction and elongation a number of times in a single spot, changing direction each time it contracts until it has described a complete circle at least once and sometimes several times. It then flattens itself out on the substratum and its outlines become very irregular (Fig. 3). Occasionally larvae were seen secreting tests attached to the surface film of the water as described by Wright in 1859. This flattened stage lasts for several minutes, after which time it is difficult to dislodge the organism from the slide even with a fairly strong stream of water. It is at this time that a broad layer of cement substance is being secreted which will serve to attach the test to the substratum. During this stage and for the entire time that the test is being secreted the anterior end of the animal is raised above the rest of the body.

Having attached itself firmly, the animal now assumes a more regular ovoid form, becoming thicker and rounder (Fig. 4). Soon granules may be seen around the body among the cilia. These collect all around the periphery and harden to form the bottle-shaped part of the test.

Figs. 1–4. *Folliculina aculeata.* × 300.

Fig. 1. Late division stage. Macronucleus not yet completely divided.
Fig. 2. Free-swimming larva.
Fig. 3. Larva flattened out on the substratum during the process of cement secretion.
Fig. 4. Beginning of the secretion of the test.



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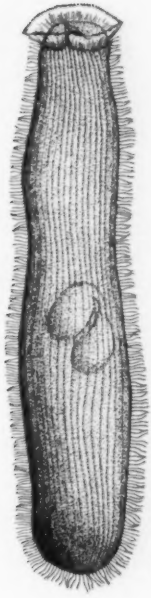

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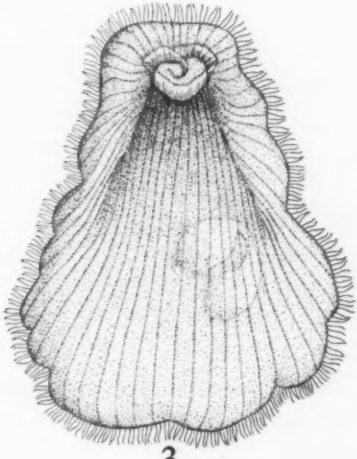

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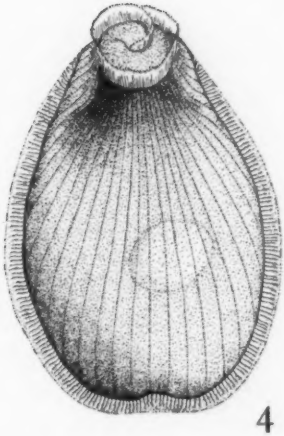

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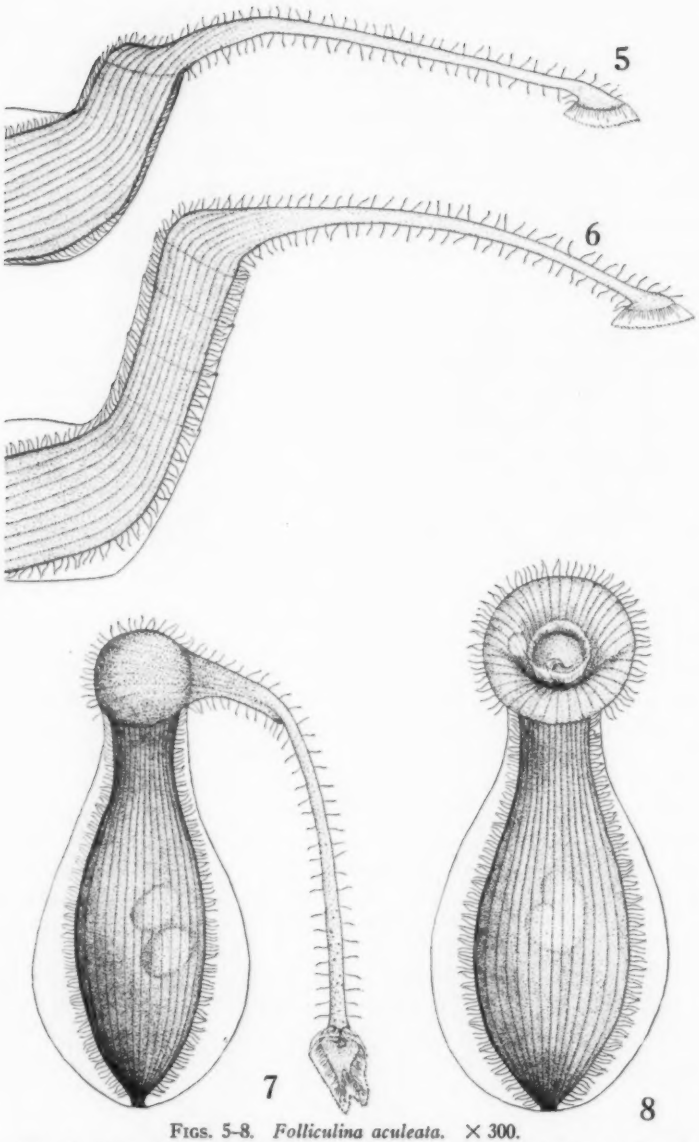

FIGS. 5–8. *Folliculina aculeata.* × 300.

FIG. 5. Side view of larva with long proboscis at the beginning of secretion of the neck of the test.

FIG. 6. Same as Fig. 5. Neck secretion nearly completed.

FIG. 7. Same as Fig. 5. Top view.

FIG. 8. Beginning of secretion of the collar of the test.

The upraised anterior portion of the body secretes the base of the neck of the test. As the formation of the body of the test is completed, the anterior end of the animal elongates to form a proboscis-like projection

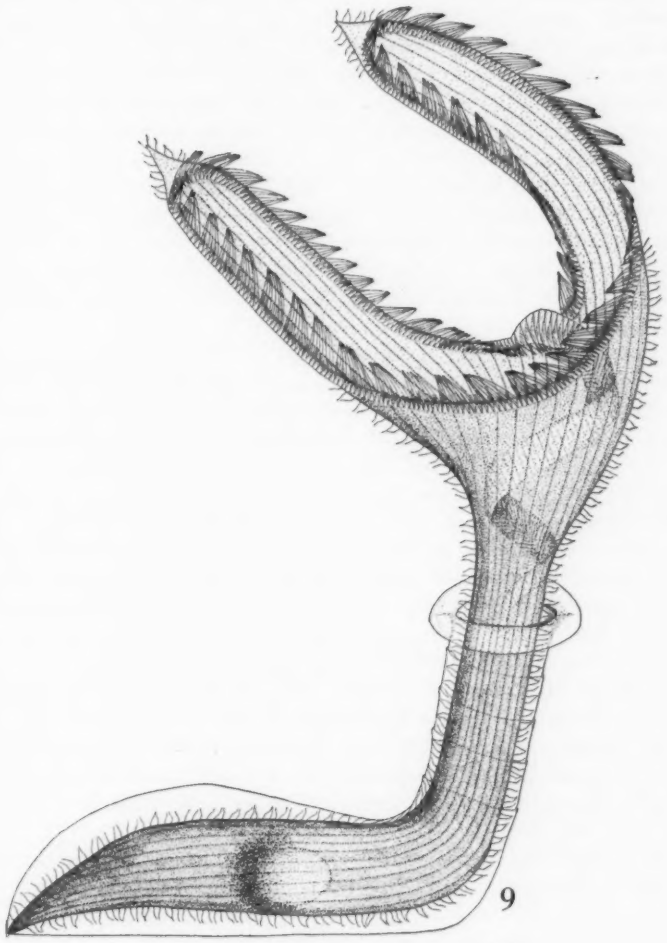

FIG. 9. Adult *Folliculina aculeata*. × 300.

of about the same length as the body. Upon the end of this proboscis the membranelles are borne (Figs. 5, 7). The proboscis is seen to sweep about in circles and while it is present the neck of the test becomes

longer and the spiral rings are laid down (Fig. 6). This stage is observed about 50 to 75 minutes after the larva has become attached and lasts about 70 to 100 minutes.

It is difficult to state whether or not this stage has previously been described. Penard (1919) states that Lachmann observed the fixation of a larva of *F. elegans* "after which . . . at the anterior end a membranous extension appeared, which I should be disposed to consider as moribund phenomena" (p. 312). Upon referring to the original paper this stage is described as presenting an "épanouissement membraneux" (p. 219). This seems clearly to refer to the next stage to be described in this cycle. It is improbable that this is an abnormality in the development, since this stage was observed in every larva whose development was followed. In the metamorphosis of *F. producta* and *F. simplex* this stage is apparently lacking (Andrews, 1923; Fauré-Fremiet, 1932). The figures of Fauré-Fremiet (1936) cannot be definitely identified with this stage.

Upon completion of the tubular part of the neck of the test, the proboscis is retracted and a double fold of cytoplasm is extended around the opening to form a collar (Fig. 8). During the 55 to 115 minutes that this stage lasts the collar of the test is secreted. When this has been finished the cytoplasm frees itself from the rim of the collar and the ragged edges are withdrawn into the test. In this contracted state the animal remains for from 3 to 5 hours. The changes occurring during this time are described in detail by both Andrews and Fauré-Fremiet and seem to be essentially similar in all forms. At the end of this period the animal protrudes from the test the long peristomeal lobes characteristic of the adult form (Fig. 9) The entire process of test secretion and morphogenesis may take from 4½ to 8¾ hours.

A single small larva of a yellow color, probably *F. viridis,* was discovered on the slide in the early stages of test secretion and was followed through to the adult stage. The development followed that of *F. aculeata* and *F. elegans,* although the proboscis was relatively shorter than in these species and the peristomeal lobes of the adult were smaller and more rounded.

I acknowledge gratefully the advice and encouragement of Dr. George W. Kidder.

Summary

1. Test secretion and metamorphosis in *Folliculina aculeata* and *Folliculina elegans* are described.

2. These processes may be divided into the following six stages:

a. The free-swimming stage.
b. The stage of cement secretion.
c. The secretion of the body of the test.
d. The secretion of the neck of the test, during which process the animal puts forth a long, proboscis-like projection. This stage is, as far as is known, peculiar to *F. aculeata, F. elegans* and *F. viridis* and has not been described before in detail.
e. The secretion of the collar of the test.
f. Formation of the peristomeal lobes characteristic of the adult.

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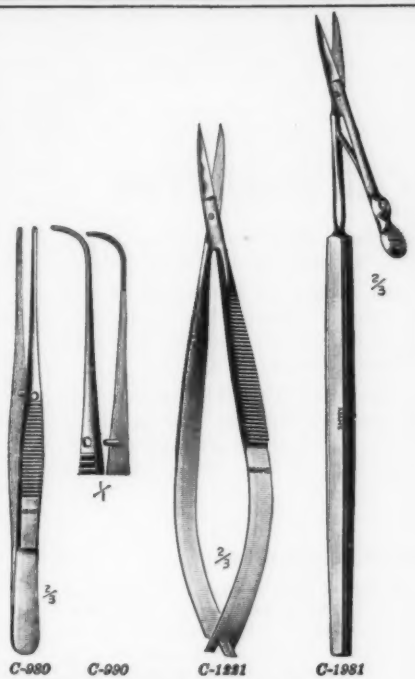

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